Glutathione S-Transferases in the Adrenal Cortex.

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Declaration of Originality

I declare that the work presented within this thesis, unless stated otherwise, is my own.

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December, 1991.

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whose continuous support, encouragement
and faith in my ability made the end result possible.

Abstract of Thesis

Data available prior to this thesis had shown that, of all bovine organs examined, the adrenal cortex exhibited the second highest level of glutathione S-transferase (GST) expression behind the liver. This finding, along with increasing evidence implicating the importance of GST in endogenous detoxification processes, formed the basis for a further extensive investigation of the GST isoenzymes expressed by the adrenal cortex.

Investigation of the GST isoenzymes expressed by a number of different bovine organs using affinity chromatography on S-hexylglutathione-Sepharose 6B (S-hexG-Ag) revealed a marked organ-specific distribution of these enzymes. Bovine adrenal cortex, in particular, expressed isoenzymes from each GST class, as determined by immunoblotting experiments. GST activity determinations of these enzyme pools using a number of model substrates revealed the bovine enzymes to possess a specificity distinct to that of rat and human GST.

Isoelectric focusing of the bovine adrenal cortex isoenzymes showed them to possess pI values similar to those found in other species. The affinity-purified mu- and pi-class isoenzymes were resolved using anion-exchange chromatography, followed by reverse-phase hplc. Using this approach, at least 3 mu-class GST subunits and 1 pi-class GST subunit were identified. Ion-exchange chromatography failed to resolve the affinity-purified alpha-class GSTs, and reverse-phase hplc analysis resolved 2 polypeptides, designated Ya_1 and Ya_3 respectively.

Analysis of the protein that failed to bind to the S-hexG-Ag column revealed that about 35% of GST activity remained in this fraction. Application of this material to glutathione-Sepharose 6B (GSH-Ag) resulted in the purification of an abundant alpha-class GST (1.3% total cytosolic protein). This GST was found to exhibit marked peroxidase and Δ^5-ketosteroid isomerase activities, in addition to high activity with 4-hydroxynonenal. SDS/PAGE analysis revealed 2 distinct polypeptides of Mr 25900 and 26500, the former being equivalent to the Ya_3 subunit purified on S-hexG-Ag, and the latter named Ya_2. Ion-exchange chromatography of the GSH-Ag purified alpha-class GST isoenzyme pool resulted in a complex picture, suggesting there to be at least 3 distinct subunits in this pool.

Both liver and testes displayed a similar high level of alpha-class GST as the adrenal cortex. The liver showed significantly higher levels of expression than the equivalent enzymes in the adrenal cortex, whereas the testes showed values comparable with the adrenal cortex.

An abundant alpha-class GST was also purified from human adrenal cortex that possessed properties that were similar to the bovine enzyme(s). Unlike the bovine GST, the corresponding human alpha-class GST bound to S-hexG-Ag, although displayed both marked peroxidase and Δ^5-ketosteroid isomerase activities. Both SDS/PAGE and reverse-phase hplc analyses established this abundant alpha-class GST in human adrenal cortex to be equivalent to the human liver GST B_1B_1 enzyme.

The physiological significance of such high levels of alpha-class GSTs with similar catalytic properties in bovine and human species, and their possible functions in the adrenal cortex, are discussed.

Experiments involving primary cultures of bovine adrenocortical cells revealed the steroidogenic agonist, ACTH, to cause a significant decrease in GST activity. Immunoblotting revealed a corresponding decrease in the Ya_1 subunit. Angiotensin II did not cause any significant change in GST expression.

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Chapter 1: INTRODUCTION.

(1) Introductory Remarks

Lipid peroxidation is the process whereby lipids of cellular membranes are oxidised by molecular oxygen to hydroperoxides and other products, forming one of the ways in which oxygen is toxic to living organisms. Because molecular oxygen is used in steroid biosynthesis, lipid peroxidation and other aspects of oxygen toxicity are major problems for steroidogenic cells (**Hornsby & Crivello, 1983 a,b**).

The adrenal cortex is rich in cytochrome P450 enzymes which are involved in steroid hormone synthesis (**Hall, 1987**). The catalytic actions of cytochrome P450 can result in the production of highly-reactive intermediates and toxic oxygen species that may pose a serious threat to the adrenocortical cell. Oxidative stress that arises during steroidogenesis may be corrected by each of the two possible forms of glutathione peroxidase, one belonging to the glutathione S-transferase enzyme family. Whilst the endogenous role of glutathione S-transferases (GSTs) in these cells is not clear, they may be involved in combating the oxidative stress prevailing during adrenocortical steroidogenesis. This is substantiated by the observations that several compounds generated in the process of lipid peroxidation serve as substrates for GST (**Ketterer *et al.*, 1987, 1990**; **Jensson *et al.*, 1986**; **Ålin *et al.*, 1985**).

It is the aim of this introductory chapter to review the current knowledge of GSTs, and provide a brief overview of the functions of the adrenal cortex. Finally, the various aspects of toxicity in the adrenal cortex will be discussed, including the reasons for studying GST expression in this organ.

(2) Enzymes of Detoxication

Cells are continually being exposed to foreign substances, also called xenobiotics, that are not essential for their normal metabolism. Continued exposure to such potentially harmful compounds can induce many serious pathological conditions, and it is therefore not surprising that the cell retains specific systems for metabolising these substances. Many of these toxic substances are hydrophobic, and it is the so-called "enzymes of detoxication" in the cell which

render them water-soluble and therefore ready for excretion. Detoxifying enzymes are widely distributed in mammalian cells with a range of different specificities and functions, and a common feature is their broad substrate specificity which make them capable of metabolising an enormous number of substances.

The different detoxication enzymes frequently co-operate in the process of converting hydrophobic xenobiotics into compounds that are water-soluble. It is recognised that there are two phases in the metabolism of foreign substances, enabling the division of detoxication enzymes into two groups: phase I and phase II (see Figure 1a). During phase I metabolism, one or more polar groups are introduced into the parent molecule, making it sufficiently electrophilic to serve as a substrate for the phase II enzymes. Phase I enzymes include a family of proteins called cytochrome P450s which catalyse the incorporation of an oxygen atom into the xenobiotic by a process of mixed-function oxidation; the product resulting from the action(s) of cytochrome P450 may then undergo further metabolism **(see reviews by White & Coon, 1980; Guengerich, 1990)**. The general reaction catalysed by cytochrome P450 enzymes can be presented as follows:-

$$R + NADPH + O_2 + H^+ \dashrightarrow ROH + NADP^+ + H_2O$$

where R is a lipophilic substrate and NADPH is an electron donor. Many distinct cytochrome P450 isoenzymes have been described, and to date more than 70 different genes coding cytochrome P450s from various species have been identified **(Nerbert & Gonzalez, 1987; Nerbert *et al.*, 1989)**. Thus, the cytochrome P450s are a diverse multi-gene family of membrane-bound enzymes which can metabolise xenobiotics. In addition to their role in drug metabolism, certain cytochrome P450s can metabolise endogenous substrates such as steroids **(Hall, 1987)**. Although a primary purpose of cytochrome P450s is to generate an electrophilic moiety for the phase II enzymes, their metabolism of foreign compounds can potentiate the detrimental effects of an inert compound by activating it to a reactive, toxic intermediate (see Fig. 1a). This particular aspect of cytochrome P450 activity, as well as their more general characteristics, has been the subject of several reviews **(Wolf, 1986; Nerbert & Gonzalez, 1987)**.

The products of cytochrome P450-catalysed reactions are often further metabolised by

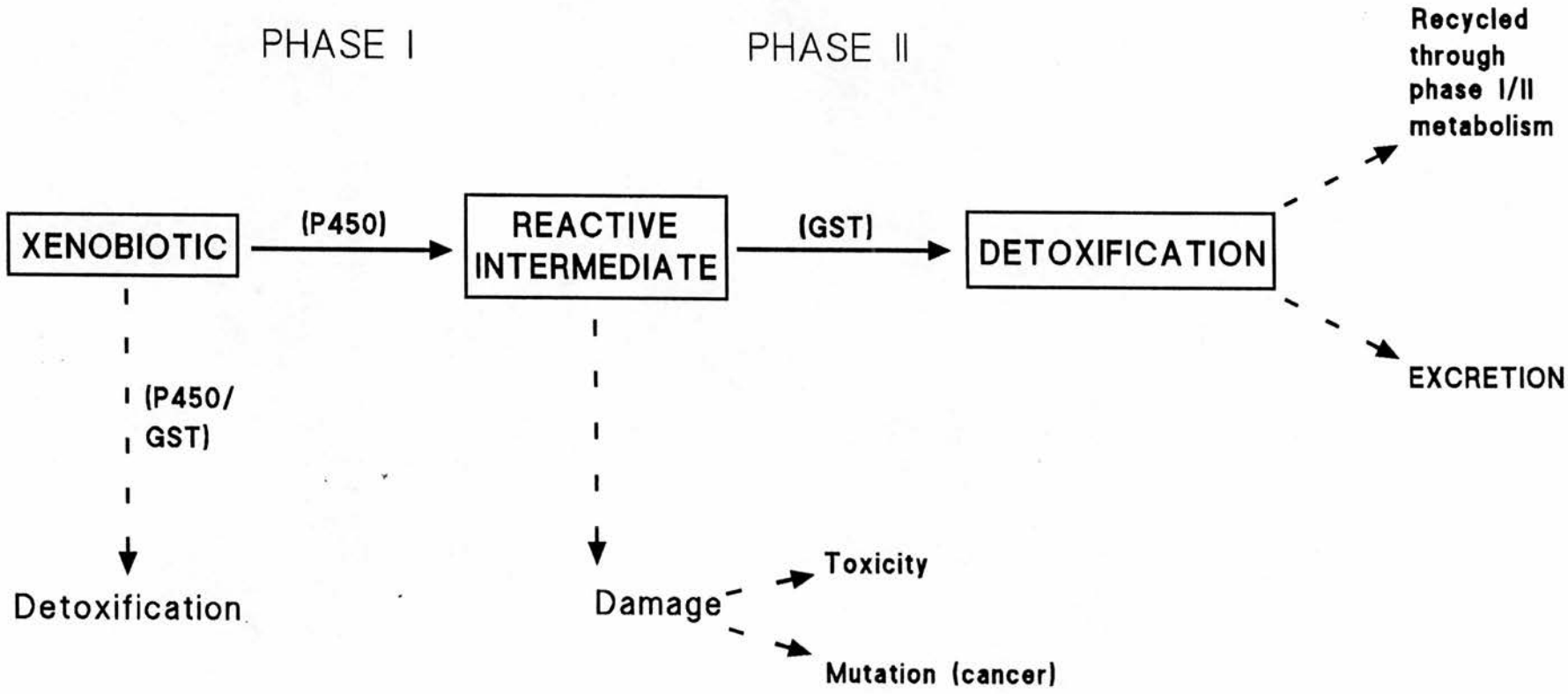


Figure 1a: Phases I and II During the Metabolism of Xenobiotics.

phase II enzymes. The actions of phase II enzymes result in the electrophilic species produced by phase I reactions being rendered more hydrophilic by conjugation and/or hydrolysis. The products of phase II metabolism are subsequently ready for excretion and/or can be re-cycled through the cytochrome P450-catalysed phase I system and/or other phase II enzymes (Fig. 1a). Examples of phase II enzymes are glutathione S-transferase, epoxide hydrolase, sulphotransferase, UDP-glucuronyltransferase and DT-diaphorase, all of which have been extensively reviewed: sulphotransferase - **Mulder, 1981**; microsomal epoxide hydrolase - **Siedegard & De Pierre, 1983**; cytosolic epoxide hydrolase - **Meijer & De Pierre, 1988**; DT-diaphorase - **Ernster, 1987**; UDP-glucuronyltransferase - **Burchell *et al.*, 1987**; glutathione S-transferase - **Mannervik & Danielson, 1988**. Throughout the course of this thesis the functions of the glutathione S-transferases, a phase II enzyme family, will be discussed.

3. Glutathione S-transferase

(a) Overview

The glutathione S-transferases (GST; EC 2.5.1.18) catalyse the conjugation of reduced glutathione (GSH) to potentially toxic electrophilic compounds, rendering the electrophile less harmful and more easily excretable by providing an appropriate substrate for mercapturic acid synthesis **(Chasseaud, 1976)**. The multiple forms of this enzyme are able to catalyse the conjugation of GSH to a wide range of electrophilic, hydrophobic compounds. GSTs are frequently expressed at high levels in cells and therefore their relatively low substrate specificity and catalytic efficiency are compensated by such abundant expression. For example, the GSTs make up between 3 and 5% of the cytosolic protein in rat liver, although at least two forms are microsomal (microsomal GST - **Morgenstern *et al.*, 1983, 1990;** leukotriene C_4 synthetase - **Soderstrom *et al.*, 1988**) which have little in common with the cytosolic forms except their specificity for GSH. Such abundant expression may be necessary for one of the main functions of the GSTs, which is to protect cells against the potentially toxic effects of both exogenous and

endogenous compounds.

The multiple isoenzymes of GST are thought to have evolved from a common ancestral gene which has diverged during evolution **(Mannervik *et al.*, 1990)**. The different forms of the enzyme have been subdivided into four classes (alpha, mu, pi and theta) on the basis of their substrate specificities, inhibition properties, primary structure and immunochemical cross-reactivity. Although the different classes of GST have broad overlapping substrate specificities, each of these classes displays certain characteristic specificities, which help greatly in their classification **(Mannervik & Danielson, 1988)**. The cytosolic enzymes are dimers comprised of subunits of Mr ranging from 27500 (Yc subunit) to 24800 (Yf subunit) from the same class, and several nomenclature systems have been developed to describe the different GST forms (see later in chapter). The GSTs have been studied in a large number of species, as well as in many different organs within each species **(Mannervik *et al.*, 1985; Ketterer, 1986; Corrigall & Kirsch, 1988; Listowsky *et al.*, 1990)**. In addition to being expressed in all tissues studied to date, each organ possesses its own distinctive isoenzyme profile. This presumably reflects the different metabolic functions and physiological stresses encountered by different organs.

Currently efforts are being made to clone the cDNAs encoding different GSTs and investigate the flanking DNA sequences responsible for the regulation of expression of the various GST classes **(Pickett & Lu, 1989; Daniel, 1989; Muramatsu *et al.*, 1990; Rushmore & Pickett, 1990 a,b; Rushmore *et al.*, 1991)**. This type of work is of particular importance in determining the mechanisms whereby GSTs contribute to drug resistance (as reviewed by **Hayes & Wolf, 1990)** In this work attempts have been made to correlate GST levels in cells with resistance to different drugs (especially anti-cancer drugs), and results have revealed that increased resistance to cytotoxic insult can be accompanied by over-expression of GST enzymes **(Black *et al.*, 1990; Wolf *et al.*, 1990)**.

A related area of study has been the involvement of GSTs in carcinogenesis **(Mantle *et al.*, 1987)**; during carcinogenesis the pi-class GST has been reported to be expressed at elevated levels in tumour tissues and pre-neoplastic lesions **(Sato *et al.*, 1984)**. Further studies have investigated the potential use of plasma GST pi measurements as a tumour marker for diagnosing

or monitoring the progression of certain cancers **(Howie, 1990).** A potential protective role for mu-class GST has been suggested from studies revealing a correlation between the absence of an isoenzyme(s) and an increased risk of lung cancer amongst smokers **(Siedegard *et al.*, 1986, 1990)**. In studying the clinical significance of expression, methods for measuring GST levels by radioimmunoassay (RIA) have been developed. In this way, GST measurements in plasma have been shown to serve as a sensitive marker of liver damage in humans **(Beckett & Hayes, 1984, 1987; Beckett *et al.*, 1985a)**. Specific use of the RIA has been made to monitor acute liver damage, for example, following paracetamol poisoning **(Beckett *et al.*, 1985b)**, alcoholic ingestion **(Hayes, PC *et al.*, 1990)** or halothane anaesthesia **(Hussey *et al.*, 1988)**.

The GSTs are also known to participate in the synthesis of biologically-active molecules such as leukotrienes and prostaglandins, an aspect which will be discussed later in this chapter. In the context of endogenous roles for GSTs, it is now known that several isoenzymes have the capacity to bind non-covalently many lipophilic compounds, including bilirubin **(Litwack *et al.*, 1971)**, steroid hormones **(Maruyama & Listowsky, 1984; Homma *et al.*, 1986)**, thyroid hormones **(Ishigaki *et al.*, 1989)**, and neurotransmitters **(Abramowitz *et al.*, 1988)**. The GSTs have subsequently been implicated as intracellular storage sites or transport proteins for hydrophobic compounds including both xenobiotics and endogenous metabolites. Although the actual binding affinities of most ligands to GST are relatively low, the high levels of these proteins in cells must be taken into account when considering the physiological relevance of this binding.

There is now some evidence to suggest that these enzymes can also form reactive conjugates. These active GSH conjugates appear to be usually formed from halogenated hydrocarbons whereby potentially harmful molecules are produced which are able to react with both proteins and DNA, causing cell damage and mutagenicity **(Igwe, 1986)**.

3.(b) Historical Background

The first report of catalytic activity, which is now known to be attributable to GST, appeared in the late 19th century when independent work by **Baumann & Preusse (1879)** and **Jaffe (1879)** demonstrated that monohalogenobenzenes were excreted in urine as unstable N-

acetylated S-substituted cysteine derivatives. These compounds, subsequently called mercapturic acids, have been isolated from the urine of several species **(see review by Jakoby & Habig, 1980)**. The cysteine moiety of mercapturic acids was considered to arise from a number of sources, although **Bray *et al* (1959)** were the first to establish that the cysteine was derived from the tripeptide, glutathione (L-γ-glutamyl-L-cysteinyl-glycine). The first reporting of actual enzymic catalysis of glutathione conjugation was in 1961 when **Booth *et al*** demonstrated GST activity in rat liver cytosol with a number of substrates. Also in the same year, **Coombes & Stakelum** were able to show that rat liver cytosol could catalyse glutathione conjugation with bromosulphophthalein. It was subsequently shown that several types of GST activity occurred in rat liver cytosol whereby the enzyme catalysing the conjugation of glutathione with aromatic halogen and nitro compounds was different from the GST catalysing glutathione conjugation with aliphatic halogen compounds **(Johnson, 1963)**. The various forms of GST subsequently discovered were classified according to the type of reaction catalysed **(Boyland & Chasseaud, 1969)**, with four GST forms recognised: glutathione S-aryltransferase, glutathione S-alkyltransferase, glutathione S-aralkyltransferase and glutathione S-epoxide transferase. This substrate-based nomenclature system was widely adopted, despite the fact that none of the enzymes responsible for these activities had been purified. As various GSTs were isolated it became evident that separate isoenzymes had overlapping substrate specificities, and consequently this classification scheme was abandoned and alternative forms of nomenclature sought.

The development of affinity matrices such as glutathione-Sepharose 6B **(Simons & Vander Jagt, 1977)** and S-hexylglutathione-Sepharose 6B **(Guthenberg *et al*., 1979)** greatly facilitated the purification of GST and the preparation of homogenous fractions. Affinity chromatography, in conjunction with other methods such as chromatofocusing, gel filtration and ion-exchange chromatography, rapidly enabled the identification and characterisation of GST forms in a whole range of different species: bacteria **(Di Illio, 1988)**, yeast **(Tamaki *et al*., 1989)**, insects **(Jansen *et al*., 1982)**, fish **(Nimmo, 1987)**, plants **(Mozer *et al*., 1983)**, and numerous mammalian species **(Mannervik *et al*., 1985)**. Using different combinations of these chromatographic methods it

became clear that cytosolic GSTs are dimeric, each comprising two subunits which are encoded by a limited number of genes (in the rat there are at least 13 genes encoding cytosolic GST). Furthermore, it was concluded that both homo- and hetero-dimers exist, and that the large number of forms observed arise partly as the result of subunit hybridisation **(Hayes *et al.*, 1981; Beale *et al.*, 1982,1983; Mannervik & Jensson, 1982)**.

The most extensively-studied GST is that which possesses a high affinity for various organic anions. Initially, this enzyme was isolated because of its ability to bind certain carcinogens **(Ketterer *et al.*, 1967)**, cortisol metabolites **(Morey & Litwack, 1969)** and both bilirubin and bromosulphophthalein **(Levi *et al.*, 1969)**, and consequently was assigned the name "ligandin". Some cofusion existed concerning the actual properties of "ligandin" and it took another several years before it was formally identified as a GST **(Habig *et al.*, 1974)**. Further controversy existed in the following years as to the exact subunit composition of "ligandin" since different workers appeared to assign the name "ligandin" to different proteins. Consequently, the term is no longer used to describe this GST form.

A relatively large number of different GST forms have now been characterised in various species (especially in the rat and man) and these, along with the nomenclature systems developed to describe them, will be discussed later in this chapter.

3.(c) Glutathione

The multiple forms of GST catalyse the conjugation of reduced glutathione (GSH) with numerous electrophilic, hydrophobic compounds in the first step of mercapturic acid formation **(Boyland & Chasseaud, 1969)**. Glutathione is synthesised intracellularly from glutamate, cysteine and glycine via the sequential actions of γ-glutamylcysteine synthetase and glutathione synthetase to form the tripeptide, L-γ-glutamyl-L-cysteinyl-glycine (glutathione). An important aspect of glutathione metabolism is that the series of reactions catalysing the synthesis and degradation of glutathione are closely linked with its transport out of cells and transport of γ-glutamyl amino acids into cells, a process known as the γ-glutamyl cycle (**Meister & Tate, 1976**).

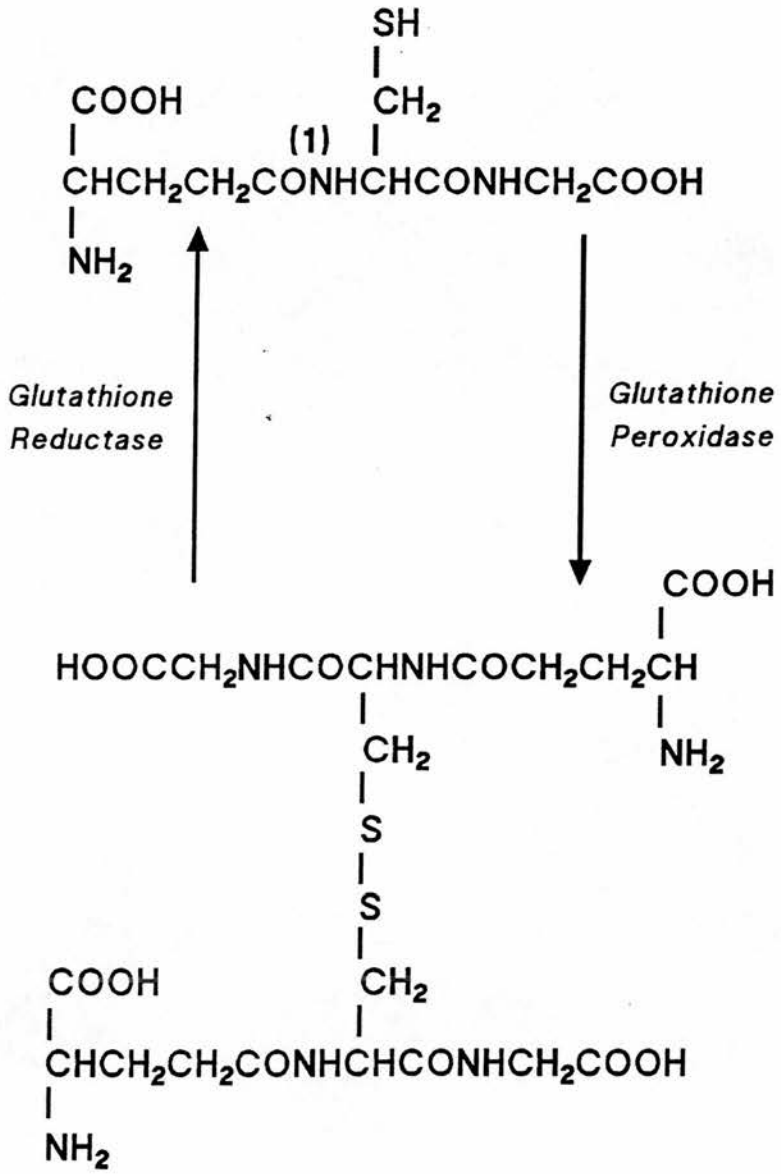
Cellular glutathione concentrations are maintained by achieving a balance between the rate of

synthesis and rate of utilisation **(Kaplowitz *et al.*, 1985)**. Glutathione can exist in the reduced (GSH) or oxidised (GSSG) form (see Fig. 1b), although *in vivo* most glutathione is present as GSH: in man, GSH levels range from 0.5 to 10 mmol/l depending upon the organ, compared to values between 4 and 10 μmol/l for GSSG **(Meister & Anderson, 1983)**. Glutathione is ubiquitous in eukaryotic cells, being the most abundant low molecular weight peptide present and consequently it has been implicated in a number of cellular functions.

Reduced glutathione (GSSG) and glutathione S-conjugates generated through the reactions of glutathione peroxidase and glutathione S-transferase have been shown to be eliminated from cardiac cells via a common export system **(Ishikawa & Sies, 1984)**. This transport system has been found to be closely-linked to the cytosolic free ATP/ADP ratio (**Ishikawa *et al*, 1986**), and there is now direct evidence for the ATP-dependence of this process in rat heart sarcolemma vesicles (**Ishikawa, 1989**). Further experiments have shown this transport system to translocate leukotriene C_4 in plasma membrane vesicles prepared from rat heart and liver (**Ishikawa *et al*, 1989**).

There are several chemical properties which make glutathione an ideal molecule for its probable main function of forming thioether conjugates with electrophilic compounds as an aid to the elimination of such compounds from the body **(Boyland & Chasseud, 1969)**. For example, the isoelectric point of GSH is pH 2.8 and therefore it will take on a net negative charge at physiological pH. The corresponding hydrophilic nature of GSH at physiological pH will increase the solubility of hydrophobic moieties to which it may become conjugated. Another important feature of GSH is the γ-glutamyl peptide bond between the N-terminal glutamate and cysteine residues (Fig. 1b). This bond cannot be cleaved by α-carboxypeptidase but can be by γ-glutamyl transpeptidase: this latter enzyme catalyses the cleavage of the glutamate residue from the glutathione conjugate, which is the second step in mercapturic acid synthesis. The low molecular weight of GSH (Mr 307.3) also ensures that the conjugate will be efficiently cleared via the biliary system **(Hirom *et al.*, 1972)**. The range of cellular functions now proposed for glutathione include involvement both as a reducing agent and antioxidant, providing a cysteine reservoir, participation in detoxification reactions for xenobiotics and numerous endogenous

Reduced Glutathione (GSH)



Oxidised Glutathione (GSSG)

(1): γ–glutamyl peptide link

Figure 1b: The Chemical Structure of Glutathione and the Inter–relationship Between Reduced Glutathione (GSH) and Oxidised Glutathione (GSSG).

compounds, requirement for prostaglandin and leukotriene synthesis, and finally possible involvement in cell cycle regulation **(Meister & Anderson, 1983; Deneke & Fanberg, 1989)**.

Of all the functions and possible roles of GSH, its involvement in the mercapturic acid pathway must be one of the most important. The conjugation of electrophilic xenobiotics with the sulphur atom of GSH by GST usually produces a decrease in the reactivity and toxicity, and an increase in the solubility, of the compound. This conjugation reaction is the first step in mercapturic acid formation (see Figure 1c) and is an essential reaction enabling the excretion of potentially toxic xenobiotics, as well as endogenous metabolites. The involvement of GSH conjugates in this process has been known since 1959 **(Barnes *et al.*, 1959; Bray *et al.*, 1959)**, and the role of GSH in the conjugation reaction catalysed by GST has been reviewed by **Boyland & Chasseaud (1969)**. In brief, thioesters formed by the conjugation reaction are converted to mercapturic acids in three separate stages (Fig. 1c). In stage 1, catabolism of glutathione conjugates usually takes place in the kidney where γ-glutamyltransferase catalyses the transfer of the γ-glutatmyl moiety to neutral amino acids or peptides. Cysteinyl-glycinase, a dipeptidase, catalyses the hydrolysis of the cysteinyl-glycine peptide bond with the release of glycine (stage 2). The cysteinyl derivative can then be converted by N-acetyltransferase to a mercapturic acid by N-acetylation with acetyl co-enzyme A (stage 3). Alternatively, the cysteine conjugate can be converted to the thiol derivative, pyruvate and ammonia by cysteine conjugate β-lyase. Importantly, the GSH conjugate formed by GST activity can be excreted directly in bile as well as undergoing further metabolism through the mercapturic acid pathway.

3.(d) Nomenclature

(i) rat GST

As mentioned earlier in the chapter, the original classification system of cytosolic GST activity that was based on substrate utilisation (as described by **Boyland & Chasseaud, 1969)** was found to be inappropriate. **Habig *et al* (1976)** purified several enzymes from rat liver cytosol, naming them **AA, A, B, C, D and E,** although the purification system adopted did not resolve all the GST forms present in this tissue and consequently the nomenclature based on this method

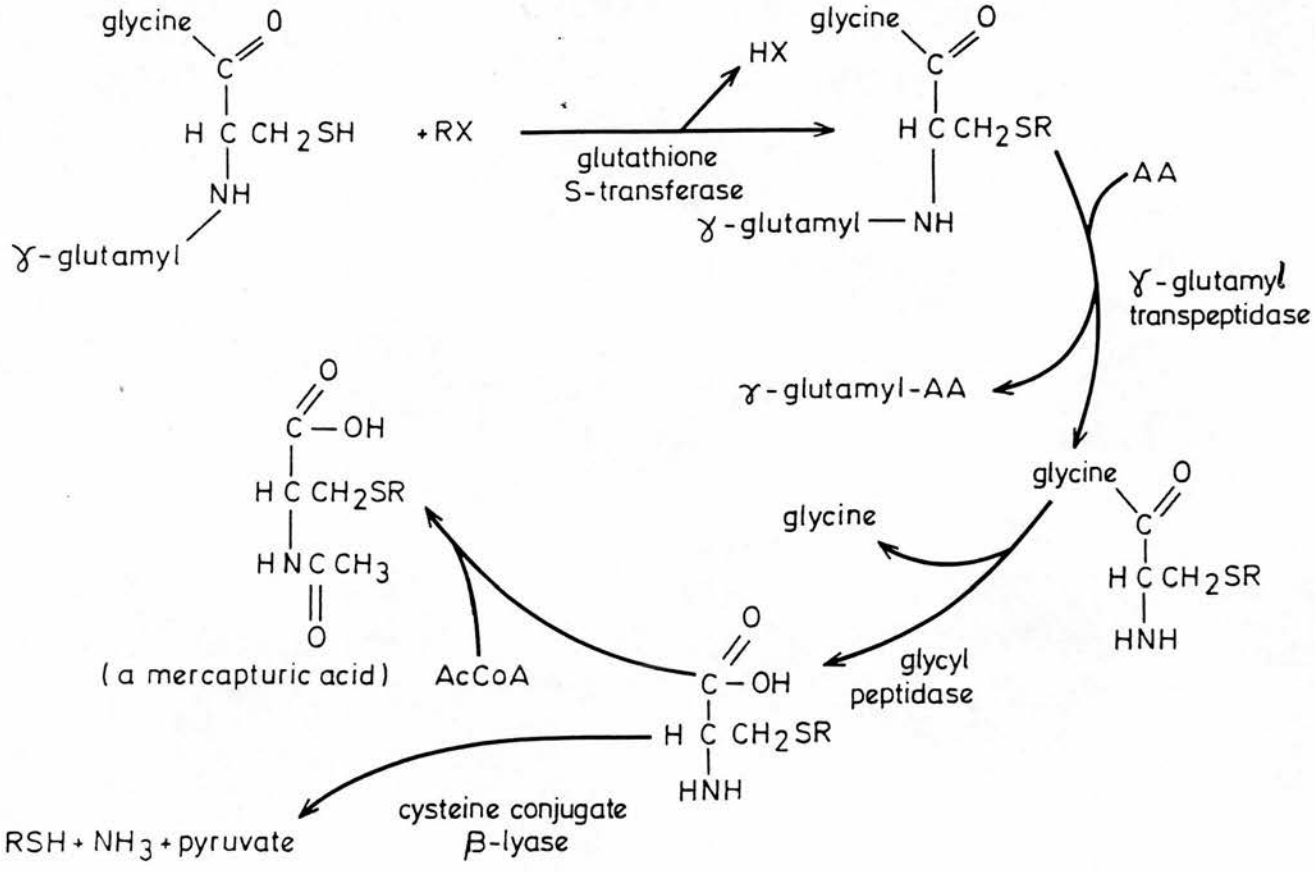


Figure 1c: The Mercapturic Acid Pathway.

was abandoned. Cytosolic GST are dimeric and it is their quaternary structure which provides the basis for the current nomenclature system. The realisation that GSTs were best described by their subunit composition resulted in the introduction of a nomenclature system which defined subunits according to their mobility on SDS/PAGE, known as the "Y" designation. Using SDS/PAGE to resolve GST polypeptides, **Bass *et al* (1977)** identified the Ya, Yb and Yc subunits from rat liver cytosol. This nomenclature system has now been extended to include further subunits described by other workers such as Yf, Yk and Yn **(Hayes, 1984, 1986)**. The SDS/PAGE system utilised by these workers is the one described by **Laemmli (1970)**, although further observations made by **Hayes & Mantle (1986a)** revealed that the amount of cross-linker incorporated in the resolving gel greatly influences the relative mobility of different subunits. The relative mobilities of the subunits on SDS/PAGE in 12.5% polyacrylamide resolving gels containing 0.32% NN'-methylenebisacrylamide is: Yf > Yk > Ya > Yn > Yb > Yc **(Hayes, 1986; Hayes & Mantle, 1986b)**. These subunits were initially grouped into three families, or classes, of GST called alpha, mu and pi, of which Ya/Yc, Yb/Yn and Yf subunits are members, respectively **(Mannervik, 1985)**. However, relatively recently a subunit has been purified from rat liver designated Yrs which does not belong to any of these three GST classes described and has been ascribed to a separate family, the theta class **(Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991)**.

Mannervik (1985) initially proposed the idea of "gene families", whereby the enzymes comprising Yf monomers, Ya and/or Yc (and now Yk) monomers, and Yb and/or Yn monomers each represent separate structural groups with members within each group deriving from the same gene family. Evidence for this theory came collectively from N-terminal amino acid sequence studies **(Frey *et al.*, 1983; Mannervik, 1985)**, peptide mapping **(Beale *et al.*, 1983; Hayes, 1984)** and immunochemical cross-reactivity **(Hayes & Mantle, 1986b)**. Work by **Beale *et al* (1982)** suggesting the Ya and Yc subunits to be coded for by two separate genes that have arisen either by gene duplication or divergence, along with similar work by **Hayes (1983)** for the mu-class GST subunits, led to the idea that the genes encoding these subunits were distant relatives of a common ancestral gene **(Mannervik, 1985)**. Important work involving *in vitro* hybridisation studies has further shown that subunits from the same, but not different, classes can

hybridise with each other **(Kitahara & Sato, 1981, Boyer *et al.*, 1983; Hayes, 1983 ,1984)**. Thus, only certain GST subunit combinations are possible, which limits GST diversity.

In summary, the GSTs can be divided into four classes named alpha, mu, pi and theta, on the basis of substrate specificities, immunochemical cross-reactivity, primary structure and inhibition properties. In general, the primary structures of GSTs show between 60 and 98% sequence homology within each class, although only 20-30% homology exists between different classes **(Mannervik *et al.*, 1990)**. For example, the homology based on amino acid sequence data for the alpha-class GST (Ya, Yc and Yk subunits) is 58%, and the mu-class GST (Yb_1, Yb_2 and Yn subunits) greater than 80%.

Considerable evidence is available now to demonstrate that some of the existing GST subunits already described exist in more than one form, and to date this has been shown to be the case for both the rat Ya and Yb subunits. For the Ya subunit, Southern blot analysis of rat genomic DNA using specific probes to 5' and 3' regions of the rat Ya gene has estimated that there are at least five Ya genes within the rat genome **(Rothkopf *et al.*, 1986)**. Two cDNA clones encoding the rat Ya subunit have been isolated which encode for polypeptides of 222 residues which differ by 8 out of these 222 residues. These clones are referred to as pGTB 38 **(Pickett *et al.*, 1984)** and pGTR 261 **(Lai *et al.*, 1984)**. **Hayes *et al* (1990)** were able to resolve two separate Ya-type subunits from rat liver which suggested distinct genetic origins by showing small differences in primary structure. These subunits were designated Ya_1 and Ya_2, and their primary structures suggested them to be encoded by the genes corresponding to the pGTR 261 and pGTB 38 cDNA clones, respectively. Of further interest here is the observation made by **Hayes *et al* (1990)** that the Ya_2 subunit in rat liver becomes preferentially over-expressed in pre-neoplastic nodules which have been induced by aflatoxin B_1. Recently, a second Yc-type subunit has been identified in rat liver cytosol, designated Yc_2, from rats fed on a diet containing the anticarcinogenic agent, ethoxyquin **(Hayes *et al.*, 1991)**. The elevated expression of this novel alpha-class GST subunit has been associated with an increased resistance to aflatoxin B_1-8,9-epoxide.

At least two distinct types of Yb subunit have been found in the rat (Yb_1 and Yb_2) which

have been shown to form dimers *in vivo* **(Hayes, 1983)**. Further *in vitro* hybridisation experiments have demonstrated that both the Yb subunits dimerise with the Yn subunit: transferases P and N described by **Hayes (1984)** correspond to the Yb_1Yn and Yb_2Yn heterodimers, respectively. Southern blot analysis has again indicated the presence of multiple genes **(Lai *et al.*, 1986)**, and cDNA clones encoding the Yb_1 and Yb_2 subunits have been isolated **(Ding *et al.*, 1985, 1986)**. From such sequences it would appear that there is at least 80% homology between the two Yb subunits.

The alpha, mu and pi classes of GST have been well-described, but more recently a fourth class of GST has been discovered **(Hiratsuka *et al.*, 1990; Meyer *et al.*, 1991)** which has been called the theta class. Two members of this family, named transferase E and M **(Fjellstedt *et al.*, 1973; Gillham, 1973; Meyer *et al.*, 1984)**, have been recognised for many years. These two theta-class GST have been purified from rat liver and can be distinguished from the other GST classes by both their lack of activity towards CDNB and lack of affinity for the glutathione affinity matrices used to purify other GSTs. As yet only one of the theta class subunits from the rat has been given a "Y" nomenclature designation, called Yrs. The alternative form of nomenclature, introduced by **Jakoby *et al* (1984)**, and updated by **Meyer *et al.*, (1991)**, has described these two theta class GSTs as 5-5 and 12-12.

Table 1a illustrates the four classes of GST, describing the particular subunits assigned to each class using both the "Y" nomenclature **(Bass *et al.*, 1977)** and numerical designations **(Jakoby *et al.*, 1984)**, in addition to several other numerical systems which have been previously used. Throughout the course of this PhD thesis the "Y" designation will be used when referring to GST subunits.

(ii) human GST

Much of the early work studying human GSTs used liver as the tissue source. Like the rat, multiple hepatic forms of the enzyme were found to be expressed. These enzymes were divided, according to charge, into three groups designated as basic, neutral and acidic **(Warholm *et al.*, 1983)**, which represent the alpha, mu and pi classes respectively. **Kamisaka *et al* (1975)**

Table 1a: Alternative Nomenclatures for Rat Cytosolic GST Subunits.

GST Class	Y-designation	Numerical Designation	Other
Alpha	YaYa	1-1	Ligandin[2]
	YaYc	1-2	B[2]
	YcYc	2-2	AA[1]
	YkYk	8-8	K[2]
	Y1Y1	10-10	
Mu	Yb_1Yb_1	3-3	A[1]
	Yb_1Yb_2	3-4	C[1]
	Yb_2Yb_2	4-4	D[1]
	YnYn	6-6	N[2]
	Yb_1Yn	3-6	P[2]
	Yb_2Yn	4-6	S[2]
	YoYo	11-11	
Pi	YfYf	7-7	P[3]
Theta		5-5	E[1]
	YrsYrs	12-12	

Notes:

Y-designation based on system devised by Bass *et al* (1977).

Numerical designation proposed by Jakoby *et al* (1984).

Definition of classes by Mannervik *et al* (1985).

1- nomenclature employed by Fjellstedt *et al* (1973), and Habig *et al* (1974 and 1976).

2- nomenclature employed by Hayes *et al* (1979), and Hayes (1984 and 1986).

3- nomenclature assigned by Kitahara *et al* (1984).

provided the first definitive data on human liver alpha-class GST by purifying five basic GST forms which were defined as α, β, γ, δ and ϵ. Determination of amino acid composition and catalytic properties of these forms led **Kamisaka *et al*** to conclude that they merely represented charged isomers of a single gene product, although further gel electrofocusing (pH range 7-10) suggested that the five enzymes purified were not artefacts of the purification procedure (i.e. multiple human hepatic GST forms do exist *in vivo*). **Stockman *et al* (1985; 1987)** purified and characterised three basic GSTs from human liver consisting of combinations of B_1 and B_2 subunits, which are products of separate genes. Results from subsequent work involving amino acid sequence data **(Hayes *et al*., 1989a)** and DNA sequence information **(Tu & Qian, 1986; Rhoads *et al*., 1987)** have confirmed these observations. These basic GST, termed GST B_1B_1, GST B_1B_2 and GST B_2B_2, are also known as GST ϵ, γ and δ respectively. Both the B_1 and B_2 subunits possess similar electrophoretic properties to the Ya subunit in rat liver.

Several studies have shown alpha-class GST to be present in a number of extra-hepatic human tissues such as the kidney **(Tateoka *et al*., 1987)**, testis **(Aceto** *et al*., **1989)** and prostate **(Tew *et al*., 1987)**. Available data suggest these extra-hepatic alpha-class GST to be equivalent to those enzymes identified in liver. However, one important exception concerns a very basic GST (isoelectric point at pH 9.9) which was identified in human skin and appears to be homologous with the alpha-class YcYc homodimer in the rat **(Del Boccio *et al*., 1987)**.

In one particular study, a mu-class GST with a neutral isoelectric point was shown to occur in a population at a frequency of about 60% (**Warholm *et al*., 1980**). This enzyme was subsequently purified from human liver by **Warholm *et al* (1981)** and called transferase μ. GST μ was the first human mu-class enzyme to be purified, and further work has shown GST μ to have an allelic variant, GST ψ **(Hayes, 1989)**. These two mu-class enzymes have been shown to be catalytically similar with at least 97% identity, confirming their existence as allelic variants.

The first description of a GST with an acidic isoelectric point came from **Marcus *et al* (1978)** who purified such an enzyme from human erythrocytes. However, later work identified similar acidic proteins in different tissues, and the designation given to each enzyme depends upon the tissue from which it was purified. For example, pi-class GST has been referred to as

"GST-π" and "GST-P" when isolated from the placenta,"GST-λ" when isolated from the lung, and "GST-ρ" when isolated from erythrocytes. There has been recent controversy concerning the question of the number of distinct human pi-class GSTs that exist *in vivo*. **Howie *et al* (1988)** have shown the pi-class GSTs from different tissues to have identical isoelectric points and the same immunochemical cross-reactivity, suggesting the existence *in vivo* of only one GST pi enzyme. However, this argument has been fuelled again by **Board *et al* (1989)** who have carried out hybridisation experiments with a partial cDNA sequence of the GST pi gene to human chromosomes. These data have shown hybridisation not only with band 11q13 (a localisation previously reported by **Laisney *et al*., 1984; Islam *et al*., 1989)**, but also with bands 12q13 and 12q14, indicating the presence of a GST pi-like gene on the long arm of chromosome 12 in man. However, it is still unclear whether this hybridisation signal actually represents an active pi-class gene or merely a pseudogene.

As described for the rat, a novel class of GST has recently been described in human liver, called the theta class **(Meyer *et al*., 1991)**. Partial analysis of its primary structure has shown that subunits 5 and 12 in the rat show a high degree of homology with the human GST θ (theta), where 25 out of the 27 N-terminal amino acids are identical. However, this novel enzyme in both species shows little relationship to the alpha, mu and pi classes of mammalian GSTs.

The nomenclature used to describe the four cytosolic classes of GST found in human tissues is illustrated in Table 1b. In addition to the soluble GST enzymes, a human microsomal GST has been described **(McLellan *et al*., 1989)** which differs from the other GST classes in that it is a trimeric protein comprising three identical subunits of Mr 17 300. Also included in Table 1b is the nomenclature system developed by **Board (1981)**. Using starch-gel electrophoresis of cell extracts coupled with a GST activity stain, three separate GST loci were reported, termed GST 1, GST 2 and GST 3, which are known to encode for the mu, alpha and pi class GST, respectively. Further work identified other GST loci: GST 4 was reported in human muscle, which also showed close homology to GST 1 **(Board *et al*., 1988)**; GST 5 was reported in brain tissue **(Laisney *et al*., 1984)**, which also showed close homology to GST 1; finally, GST 6 was also reported from brain tissue **(Suzuki *et al*., 1987)** which consisted of two dissimilar subunits and

Table 1b: Nomenclatures for Human Cytosolic GST Isoenzymes.

GST Class	Isoenzyme	Mr	pI	Locus Designation[1-3]
Alpha	B_1B_1[4] (ϵ)[5]	25900	8.9	GST-2
	B_1B_2[4] (δ)[5]	25900	8.75	GST-2
	B_2B_2[4] (γ)[5]	25900	8.4	GST-2
	Skin 9.9[6]	27500	9.9	
Mu	μ[7]	26700	6.1	GST-1
	ψ[8]	26600	5.5	GST-1
	ϕ[9]	26700	4.6	
		27300	5.2	GST-4
		27500	5.9	GST-5
		26950	4.25	GST-6
		27500	4.25	GST-6
Pi	π[10]	24800	4.8	GST-3
Theta	θ[11]			

References:

(1) Board (1981); (2) Strange *et al* (1984); (3) Suzuki *et al* (1987); (4) Stockman *et al* (1985 & 1987); (5) Kamisaka *et al* (1975); (6) Del Boccio *et al* (1987); (7) Warholm *et al* (1983); (8) Hayes (1989); (9) Stockman & Hayes (1987); (10) Guthenberg *et al* (1979); (11) Meyer *et al* (1991).

showed no homology with any of the other GST classes.

An important aspect of the work by **Board (1981)** was that it reported for the first time a high degree of heterogeneity between individuals. This polymorphic expression of GSTs in humans has subsequently been characterised more fully, and is a feature which must be taken into account when studying GST expression in isolated tissues.

(iii) bovine GST

The bovine GSTs have not been well-characterised, which is reflected in the lack of a proper nomenclature system to define individual bovine GST subunits. Curiously enough, unlike the rat and human species where almost all of the initial studies focussed on hepatic GST expression, much of the early work on bovine GSTs involved studies of the different tissue-types making up the bovine eye. The earliest documented report on bovine GSTs was by **Saneto *et al.*, (1980)** who discovered both cationic and anionic forms to exist in the bovine ocular lens. Subsequent work by **Saneto *et al* (1982)** on the bovine retina, using a range of methods including gel filtration, affinity chromatography and isoelectric focusing, revealed two near-neutral forms (isoelectric points of 6.34 and 6.87) which also displayed both types of glutathione peroxidase activity. However, the first real comprehensive study of GSTs in the bovine eye came from **Ahmad *et al* (1988)**, who investigated GST expression in the lens, cornea and retina. Estimations of isoelectric points, along wih catalytic data, amino acid sequence comparisons, and immunochemical cross-reactivities using antibodies raised against the different GST classes, collectively indicated the differential expression of the three GST classes (alpha, mu and pi) in each of the eye tissues studied. Whereas the lens was found to express the mu class GST only, the cornea expressed subunits from both the alpha and pi classes, and the retina the mu and pi classes. In contrast to these results, **Ahmad *et al* (1989)** revealed in a subsequent study of bovine iris and ciliary body tissues that all three classes of GST were present in each of these two tissues. Recent work involving the bovine ciliary body has resulted in the apparent purification of an enzyme which resembles the type I glutathione peroxidase enzyme, although initial results have indicated this protein to consist of four identical subunits without the presence of selenium

(Shichi, 1990; Shichi & Demar, 1990).

Some work has been done using bovine organs other than the eye. For example, **Asaoka (1984)** was able to purify mu-class GSTs from bovine liver using the triazine dye agarose, Orange A, followed by DEAE-Sephacel. However, later studies have shown this organ to contain other classes of GST **(Hayes *et al.*, 1989b)**, and the shortfall of **Asaoka's** method to identify other classes of GST could be attributed to the triazine dye agarose since this is known to be selective in its binding of different GST subunits. Other bovine tissues studied include the brain, in which two non-identical GST subunits of Mr 22 000 and 24 000 were isolated **(Young & Briedis, 1989)**; isoelectric points of these subunits (7.39 ± 0.02), along with catalytic data, again suggest the purification of mu-class subunits. Bovine placenta has also been used as a tissue source for GST in which a pi-class enzyme has been purified to homogeneity **(Schaffer *et al.*, 1988)**. This enzyme, of Mr 23 000 and isoelectric point 6.9 ± 0.2, was used to grow tetragonal crystals which were found to be suitable for subsequent X-ray crystallographic studies. However, such structural data has not yet been forthcoming.

The effects of various inhibitors (e.g. bilirubin, thyroxine, lithocholic acid, retinoic acid and retinol) and chemical modifiers (e.g. phenylgyloxal, iodoacetamide and butadione) on bovine GSTs have also been investigated, using GSTs isolated from bovine liver **(Asaoka & Takahashi, 1989)**, bovine brain **(Young & Briedis, 1990)** and bovine placenta **(Schaffer *et al.*, 1988)**. Such investigations have involved the monitoring of the enzyme-catalysed reaction between CDNB and GSH, and results with bovine brain GST have shown bilirubin to be the most potent inhibitor **(Young & Briedis, 1990)**.

Several studies have been carried out to investigate the distribution of the various GST subunits in different bovine tissues **(Aceto *et al.*, 1986; Hayes *et al.*, 1989b)**. The results have suggested the differential expression of the alpha, mu and pi GST classes in different tissues. Perhaps the most comprehensive study of this kind to date has been carried out by **Hayes *et al* (1989b)** who used both single-step and gradient elutions from the S-hexylglutathione-Sepharose 6B affinity matrix, as well as analysing the cytosolic fractions from each organ. Catalytic activities of the affinity-purified GSTs using a range of model GST substrates, in addition to immunoblotting

using antibodies raised against both rat and human GSTs, collectively revealed marked differences in the GST isoenzymes expressed by different tissues. The liver was found to express the highest GST levels of all the tissues studied, although the adrenal cortex showed the second highest level of GST expression. This result, emphasising the potential importance of GSTs in adrenocortical function, was the starting point for the work described in this thesis. This aspect will be discussed in much more detail towards the later part of this chapter.

2.(e) Catalysis

(i) Xenobiotic Substrates

The reaction catalysed by GST, in which electrophilic compounds are conjugated with GSH, is thought to involve interaction of each substrate with a substrate-binding site on the enzyme. In this model, each subunit contains an active centre which is composed of two binding sites, one with a high affinity for GSH, and the other a hydrophobic pocket which binds the second electrophilic substrate **(Jakobson *et al.*, 1977)**. The increased rate of GSH conjugation with electrophiles effected by the GST enzyme is thought to be due to a simple proximity effect since the rate of enhancement of the reactions catalysed is not great, and many of the GST-catalysed reactions are able to proceed non-enzymatically **(Jakoby, 1978)**.

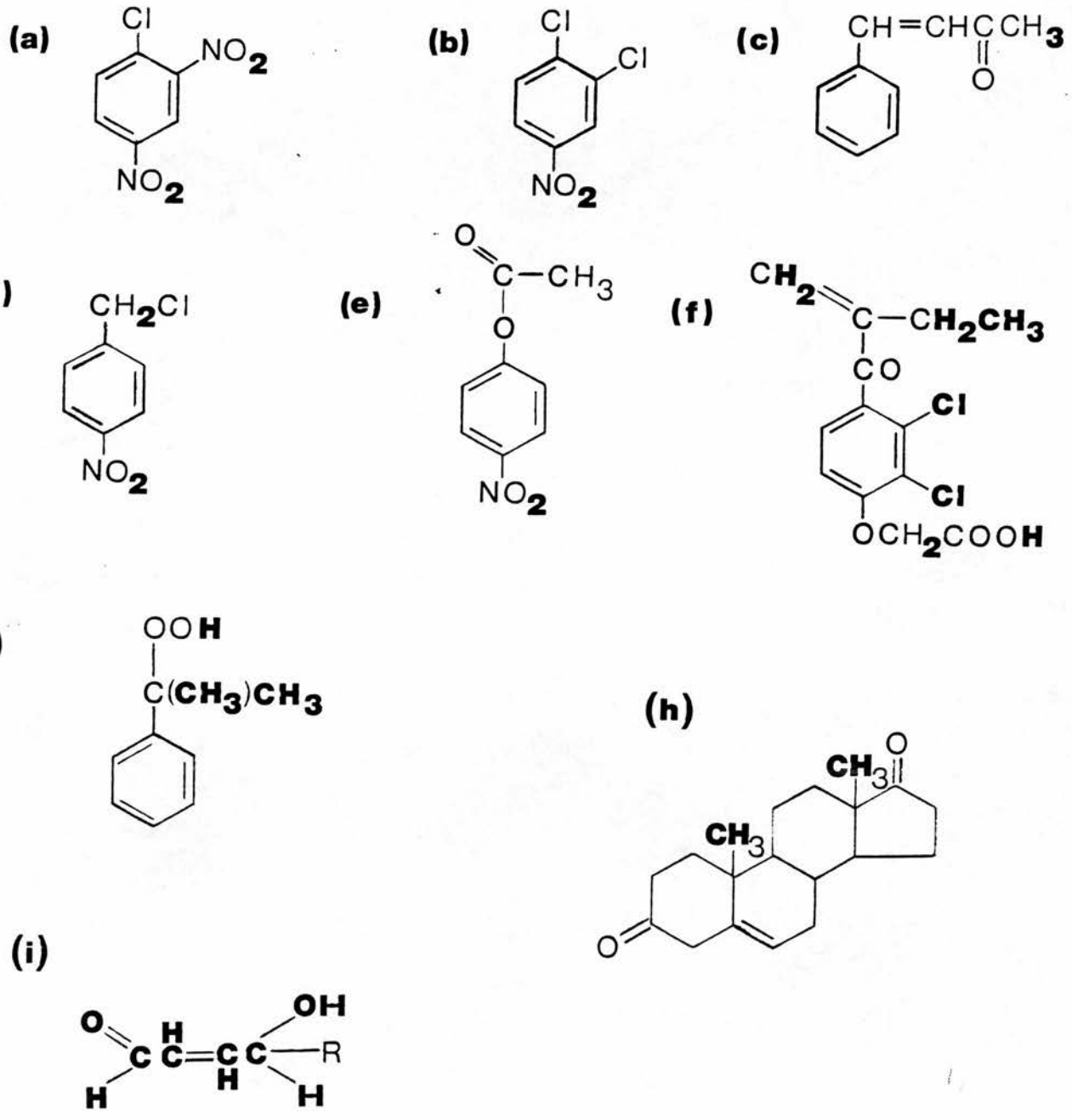
The GSTs demonstrate several types of catalytic activity with a large spectrum of structurally diverse compounds as substrates. The reactions catalysed by GSTs generally involve nucleophilic attack of GSH on various electrophiles, with the electrophilic centres consisting of either carbon, nitrogen, sulphur or oxygen atoms. Reactions involving carbon at the electrophilic centre, in which GSH is conjugated with a substrate by forming a thioether linkage, include substitution of a halogen leaving group (e.g. 1-chloro-2,4-dinitrobenzene, CDNB), addition reactions with epoxide groups (e.g. benzo[a]pyrene-4,5-oxide), addition reactions with activated alkenes (e.g. ethacrynic acid; 4-hydroxyalkenals), thiolysis reactions (e.g. p-nitrophenyl acetate), Δ^5-3-ketosteroid isomerisation (e.g. Δ^5-androstene-3,17-dione) and prostaglandin isomerisation (e.g. prostaglandin H_2). These reaction mechanisms have been reviewed by **Douglas (1987)**. Reactions displacing molecules at atoms other than carbon have also been described. One such

example is with organic nitrates, where the GSH attacks the electrophilic nitrogen, and another with organic thiocyanates, where GSTs catalyse the nucleophilic attack of GSH on the sulphur atom. Reactions involving oxygen at the electrophilic centre include the reduction of organic peroxides by alpha-class GSTs **(Prohaska & Ganther, 1977)**. This activity is referred to as selenium-independent, or type II, glutathione peroxidase, which distinguishes it from the activity catalysed by the seleno-enzyme, or type I, glutathione peroxidase **(Burk *et al.*, 1978).**

Some of the substrates included in the above reaction mechanisms, and also other known GST substrates, are illustrated in Figure 1d. Many of the conjugation reactions of electrophiles (such as those shown in Fig. 1d) with GSH can be followed spectrophotometrically at defined wavelengths, and consequently rates of reactions using different substrates with different GST isoenzymes can be estimated. Specific activity values from such experiments reveal that, although the different isoenzymes have broad and overlapping substrate specificities, isoenzymes from different classes display characteristic preferences **(Mannervik & Danielson, 1988)**. A summary of substrate specificities for the different classes of cytosolic GST in the rat is shown in Table 1c. As shown for the alpha-class enzymes, cumene hydroperoxide, Δ^5androstene-3,17-dione and 4-hydroxyalkenals have been found to show distinct activities with the Yc, Ya and Yk subunits, respectively. These substrates are relevant in the context of the adrenal cortex since they are all potentially naturally-occurring compounds in adrenocortical cells. These compounds, in addition to other endogenous substrates for GST, will be discussed more fully later in this chapter. Table 1c also illustrates the preference of the mu-class enzymes, Yb_1 and Yb_2, for the substrates 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-butene-2-one respectively, with the pi-class enzyme showing a marked preference for ethacrynic acid as a substrate.

An important substrate which has been commonly used to study GSTs is 1-chloro-2,4-dinitrobenzene (CDNB). This substrate shows activity with most of the GST classes, although significantly each class of GST displays marked differences in specific activity with this "general" substrate. Furthermore, the theta-class GST have been shown to have no activity with CDNB, and 1,2-epoxy-3-(p-nitrophenoxy)propane has been used to characterise this class of GST **(Meyer *et al.*, 1991).**

Figure 1d: Structures of GST Substrates Used in This Thesis.



Key

(a) 1–chloro–2,4–dinitrobenzene; (b) 1,2–dichloro–4–nitrobenzene; (c) trans–4–phenyl–3–buten–2–one; (d) p–nitrobenzyl chloride; (e) p–nitrophenyl acetate; (f) ethacrynic acid; (g) cumene hydroperoxide; (h) Δ^5–androstene–3,17–dione; (i) 4–hydroxyalk–2–enal.

Table 1c: Characteristic Substrates for Rat Cytosolic GST.

GST Subunit	Characteristic Substrate.
Ya	Δ^5–androstene–3,17–dione cumene hydroperoxide
Yc	cumene hydroperoxide
Yk	4–hydroxyalkenals
Yb_1	1,2–dichloro–4–nitrobenzene
Yb_2	*trans* –4–phenyl–3–buten–2–one
Yn	1–chloro–2,4–dinitrobenzene
Yf	ethacrynic acid
5*	1,2–epoxy–3–(p–nitrophenoxy) propane

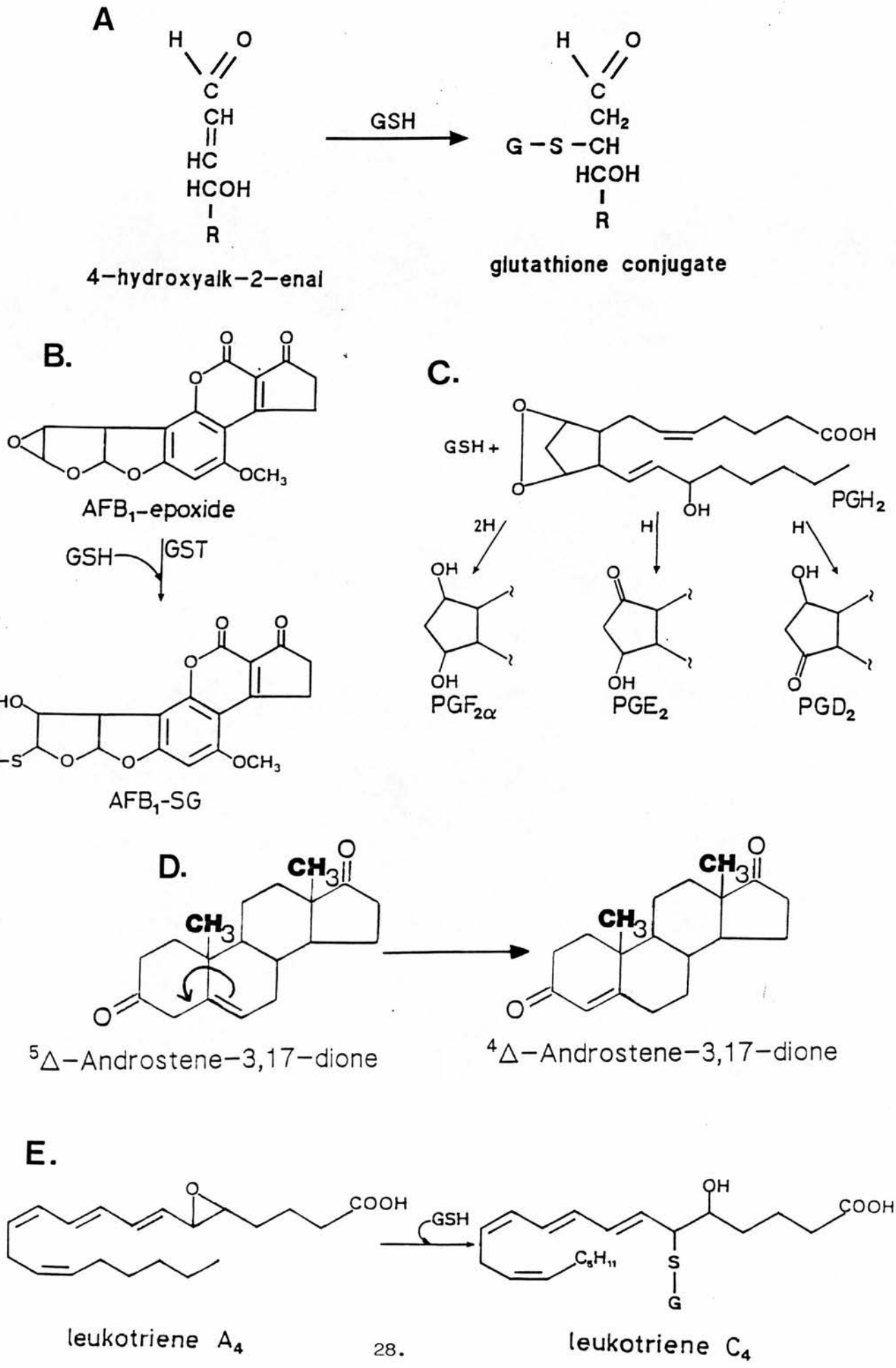
* no Y–designation has been assigned to this subunit as yet.

The different GST classes in humans have been shown to display similar substrate specifities as those in the rat **(Warholm *et al.*, 1983; Stockman *et al.*, 1987)**. In man, as in the rat, the alpha-class GST demonstrates a high specific activity for both cumene hydroperoxide and Δ^5-androstene-3,17-dione. Similarly, the mu-class GST in man, like the rat enzymes, have a high specific activity towards bromosulphophthalein and *trans*-4-phenyl-3-butene-2-one. The pi-class GST from both rat and man show a high specific activity with ethacrynic acid.

The identification of substrates which are specific for particular GST enzymes, or individual subunits, has provided a very useful method for characterising unidentified GST. In conjunction with the standard techniques of SDS/PAGE, immunoblotting and amino acid sequencing, substrate specificity allows the identity of novel GST to be established. The use of specific inhibitors has also augmented the use of various substrates to discriminate between GST subunits since, in addition to the individual subunits displaying distinctive catalytic properties towards different substrates, they also differ with respect to inhibition of activity by various compounds (for an example, see **Stockman *et al.*, 1987).** Examples of inhibitors most frequently used include cibacron blue, triphenyltin chloride, haematin, bromosulphophthalein, bilirubin, lithocholic acid-3-sulphate and S-hexylglutathione **(Tahir & Mannervik, 1986)**.

There are a number of naturally occurring epoxide substrates of GST. Cytochrome P450 can convert environmental pollutants such as the polycyclic aromatic hydrocarbons, benzo[a]pyrene and benzanthracene, into carcinogens. The most carcinogenic metabolite of benzo[a]pyrene is the 7,8-diol-9,10 epoxide which is formed by the action of a specific form of cytochrome P450, known as P448. This epoxide is acted upon by epoxide hydralase to form the 7,8-diol. All of these epoxides are substrates for GST **(Glatt *et al.*, 1983)** and the conjugates produced are excreted. The fungal toxin, aflatoxin B_1, produced by *Aspergillus flavus*, is converted by cytochrome P450 to a series of epoxides which include the carcinogen, aflatoxin B_1-8,9-oxide (Fig. 1e). This epoxide has been shown to be a GST substrate **(Coles *et al.*, 1985; Hayes *et al.*, 1991)** in certain species including the rat **(Roebuck & Wogan, 1972).** For this reason, aflatoxin B_1-8,9-oxide has been included in Table 1d (shown in the next section) which describes potential endogenous substrates for GST.

Figure 1e: Metabolism of Endogenous Substrates.



2. (e)

(ii) Endogenous Substrates and Protection Against Oxidative Stress.

Much of the emphasis regarding GST activity has concentrated on their metabolism of xenobiotics, both in their ability to detoxify harmful compounds and as tools enabling the discrimination between individual isoenzyme forms. However, there is now increasing evidence to indicate that the GSTs may also play an important part in normal cellular metabolic processes, whether providing protection against potentially toxic endogenous compounds, participating in certain normal biosynthetic pathways within the cell, or serving as binding or transport proteins in "normal" cell metabolism. Potential "natural" substrates for GSTs are listed in Table 1d.

Lipid peroxidation is a problem for steroid-synthesising tissues such as the adrenal cortex **(Hornsby & Crivello, 1983 a, b)**. When biological membranes undergo lipid peroxidation, highly reactive compounds such as epoxides, hydroperoxides and hydroxyalkenals are produced which may be potentially toxic to the cell. All of these types of molecule have been shown to be substrates for one or several forms of GST, implicating these enzymes in a protective detoxication pathway in the cell designed to remove these toxic products **(Mannervik, 1986, 1987; Ketterer *et al.*, 1987, 1990)**. As described earlier in the chapter, alpha-class GSTs display marked activities with organic hydroperoxides and hydroxyalkenals. Cumene hydroperoxide has been used extensively as a substrate to measure the selenium-independent, or type II, glutathione peroxidase activity of alpha-class GST **(Prohaska & Ganther, 1977; Burk *et al.*, 1978)**, with the Yc-type subunits in the rat showing the highest activity. The alpha-class Yk subunit has been shown to be extremely efficient in conjugating 4-hydroxyalkenals **(Ålin *et al.*, 1985; Jensson *et al.*, 1986)**, although certain other GST classes are active with this substrate, depending upon the carbon chain length **(Danielson *et al.*, 1987)**. Relatively large amounts of 4-hydroxyalkenal are known to be produced during stimulated lipid peroxidation by both rat liver microsomes **(Esterbauer *et al.*, 1982)** and isolated hepatocytes **(Poli *et al.*, 1985)**, and their conjugation to GSH would appear to be an important *in vivo* detoxication step (see Fig. 1e for reaction mechanism).

Table 1d: Endogenous Substrates for Cytosolic GST.

Substrate	Reference
Aflatoxin B_1–epoxide	Coles *et al* (1985)
Cholesterol–α–oxide	Meyer & Ketterer (1982)
Linoleic acid hydroperoxides	Ketterer *et al* (1987)
DNA hydroperoxides	Ketterer *et al* (1990)
Thymine hydroperoxide	Tan *et al* (1986)
4–Hydroxyalkenals	Alin *et al* (1985) Jensson *et al* (1986)
Prostaglandin endoperoxides	Burgess *et al* (1987) Ujihara *et al* (1988)
Leukotriene A_4	Tsuchida *et al* (1987) Soderstrom *et al* (1988)
Epoxyeicosatrienoic acids	Spearman *et al* (1985)
Steroids	Elce & Harris (1971) Elce (1972)

There are several other products of lipid peroxidation which have been found to be substrates for GST. For example, cholesterol-α-oxide has been shown to be a good substrate for the Ya-type subunits **(Meyer & Ketterer, 1982)**, in addition to linoleate and arachidonate hydroperoxides which are thought to be substrates for the theta-class GST **(Ketterer *et al.*, 1987)**. Other compounds include arachidonic acid, which gives rise to several epoxide derivatives that have also been shown to be GST substrates **(Spearman *et al.*, 1985)**.

DNA hydroperoxides have also been shown to act as GST substrates **(Tan *et al.*, 1986; Ketterer *et al.*, 1987)**, and such studies have found highest activity with the mu-class enzymes. These results, in conjunction with the observations made by **Bennet *et al* (1986)** in which mu-class GSTs were located in rat liver nuclei, suggest that the GSTs may play a role in the protection of DNA by preventing peroxidative damage to the cell's nucleic acid. The potential importance of GST in helping combat oxidative stress is also emphasised by the recent observation that the 5' flanking region of one of the rat alpha-class GST Ya genes (i.e. that encoding the Ya_2 subunit) contains an antioxidant responsive element (ARE) which results in transcriptional activation of GST during oxidative stress (**Rushmore & Pickett, 1990 a,b; Rushmore *et al.*, 1991)** [see later].

In addition to such protective functions, the GSTs are now known to participate in the biosynthesis of biologically-active molecules, including leukotrienes and prostaglandins. Prostaglandin A_1, for example, has been shown to be conjugated with GSH by homogenous rat and human transferases **(Cagen *et al.*, 1975)**, and 15-keto-prostaglandins also form conjugates with GSH **(Chaudhari *et al.*, 1978)**. Prostaglandin endoperoxides are converted into a mixture of prostaglandins F2, E2 and D2 by the action of GST **(Hayakawa *et al.*, 1977)**, as shown in Fig 1e. Leukotrienes have also been implicated as GST substrates, especially in the synthesis of leukotriene C4 by conjugation of leukotriene A4 with GSH, as shown in Fig. 1e **(Tsuchida *et al.*, 1987)**. The rat Yb_2 subunit displays significant leukotriene C4 synthetase activity **(Mannervik *et al.*, 1984)**, as does the Yn subunit **(Tsuchida *et al.*, 1987)**.

Alpha-class GST, especially Ya-type subunits, have been shown to exhibit Δ^5-3-ketosteroid isomerase activity using Δ^5androstene-3,17-dione as the substrate **(Benson *et al.*,**

1977). This isomerisation reaction, in which a double bond at the Δ^5 position is flipped to the Δ^4 position (Fig. 1e), can be monitored spectrophotometrically and has been a useful means of characterising alpha-class subunits. This reaction also occurs *in vivo* during the synthesis of steroids and is believed to be carried out by the same enzyme which shows 3-β-steroid dehydrogenase activity (**Naville *et al.*, 1991**). The significance of Δ^5-3-ketosteroid isomerase activity, as exhibited by alpha-class GST, is not known.

Several other specific compounds arising during metabolism have been identified as substrates for GST, such as reactive intermediates of oestradiol metabolism **(Marks & Hecker, 1969; Elce & Harris, 1971)**. Here, the formation, *in vivo* and *in vitro*, of GSH-conjugates of certain steroids has implicated that reactive intermediates of oestradiol-17β **(Jellinck *et al.*, 1967)** and 2-hydroxyoestradiol-17β **(Elce, 1972)** as possible substrates for GST. Quinones may also represent a group of reactive compound which may be detoxified by GST since dopaquinone , which is on the melanin biosynthetic pathway, has been found conjugated to GSH in humans suffering from malignant melanoma **(Agrup *et al.*, 1977)**.

As well as their catalytic activities towards endogenous compounds, the binding of non-substrate ligands may also be of importance. This has been discussed more fully earlier in this chapter, though suffice it to say here that the physiological role of such binding remains unclear, although storage, transport and detoxication functions have been suggested.

2.(f) Regulation of GST Expression

(i) By Xenobiotics

Hepatic GSTs can be induced by a number of compounds **(Kaplowitz *et al.*, 1975)**. Various xenobiotics have been reported to induce hepatic GSTs, such as 3-methylcholanthrene, phenobarbital, β-naphthoflavone and butylated hydroxyanisole, both *in vivo* and in isolated rat hepatocytes **(Chasseaud, 1979)**. For example, in mouse liver the anticarcinogenic antioxidant butylated hydroxyanisole (BHA) induces an alpha-class GST subunit which is not normally expressed at detectable levels **(McLellan & Hayes, 1987)**, and also induces the consitutively

expressed mu and pi class GST subunits. Similarly, the chemoprotector, β-naphthoflavone, has been shown to induce this same alpha-class GST, which has been further resolved by reverse-phase hplc into two separate non-interconvertible peaks, named Ya_1 and Ya_2 **(McLellan *et al.*, 1991)**. However, extrahepatic GST do not appear to be inducible by xenobiotics as readily as the liver enzymes **(De Pierre *et al.*, 1984)**.

The mechanism of induction by xenobiotics has been studied by examining the levels of mRNA species coding for GST subunits **(Pickett *et al.*, 1984; Ding & Pickett, 1985)**. In the current model of induction a cytosolic receptor, termed the Ah receptor (see review by **Eisen *et al.*, 1983)**, binds various planar aromatic hydrocarbons such as 3-methylcholanthrene and β-naphthoflavone. The Ah receptor-polycyclic hydrocarbon complex is then translocated to the nucleus where it interacts with positive regulatory elements called xenobiotic responsive elements, or XRE (see below), subsequently leading to the transcriptional activation of both cytochrome P450 and GST genes. One theory has suggested that polycyclic aromatic hydrocarbons (PAH) induce P450 genes initially, followed by subsequent activation of GST genes **(Talalay *et al.*, 1988; Spencer *et al.*, 1989).** The corresponding increase in protein levels due to transcriptional activation of PAH-inducible cytochrome P450 genes has the effect of raising the concentrations of reactive intermediates generated by cytochrome P450 substrates. These compounds are potential GST substrates (see part 1 of this chapter) which, according to this theory, act to induce GST. Thus, the elevation of GST levels following xenobiotic treatment is a consequence of increased cytochrome P450 activity rather than any direct effect of the Ah receptor-ligand on GST transcriptional activation.

Much of the recent work investigating the regulation of GSTs by xenobiotics has concentrated on the identification of regions of DNA which are known to cause the increased synthesis of GSTs following exposure to xenobiotics. Such studies have described two regions in the 5' flanking region of the rat GST Ya_2 subunit gene which are thought to be involved in the regulation of gene expression by planar aromatic compounds and phenolic antioxidants **(Rushmore & Pickett, 1990 a, b)**. One of the regulatory regions was found to contain a single copy of the XRE core sequence which is found in multiple copies in the 5' flanking region of the

cytochrome P450 1A1 gene. The second region was shown to contain a responsive element called the antioxidant responsive element (ARE) which was further shown to be responsive to the metabolites of planar aromatic compounds and phenolic antioxidants **(Rushmore & Pickett, 1990 a, b)**. The identification of the ARE and its response to phenolic antioxidants in the absence of a functional Ah receptor is consistent with the theory described above in which the induction of several phase II metabolising enzymes can occur through an Ah receptor-independent mechanism **(Talalay *et al.*, 1988; Prohaska & Talalay, 1988)**. Interestingly, the ARE has been found to contribute significantly to the basal level of expression of the Ya subunit gene **(Rushmore *et al.*, 1991)**. Recent studies by **Rushmore *et al* (1991)** have characterised the ARE further by defining regions of the sequence required for basal and xenobiotic-inducible expression, revealing these activities to be separate yet related through a common DNA-binding region. The ARE has also been shown to be responsive to hydrogen peroxide. In summary these studies suggest the ARE to be a cis-acting regulatory element which is responsive to oxidative stress.

2. (f)

(ii) By Hormones

Much of the work investigating the influence of hormones on GST expression has involved studies of the apparent sex-specific differences of these enzymes in rat and mouse liver (see review by **Igarashi & Satoh, 1989)**. In the rat, initial studies revealed that although CDNB activities are almost identical in hepatic GST from both sexes, the use of other substrates revealed marked differences in activities **(Igarashi *et al.*, 1984 & 1985)**, suggesting that sex differences might be reflected in different GST subunit compositions. **Igarashi *et al* (1985)** were able to show male rats to have higher levels of Yb subunits whereas the females showed higher levels of the Ya subunit. Experiments in the mouse revealed males to have a 10-fold higher level of pi-class GST than the female, a difference which only becomes noticeable following the onset of puberty, and thus implicates steroid hormones as modulators of GST levels **(Hatayama *et al.*, 1986; McLellan & Hayes, 1987).** This is given further support by results from experiments involving both castrated males, along with females who had been administered testosterone. The findings

from such studies show that castrated male mice express low pi-class GST levels (similar to those found in the female) and that testosterone treatment of the females produced the male phenotype with elevated pi-class GST levels **(Hatayama *et al.*, 1986)**. Results from such studies suggest that sex steroids (especially testosterone) are involved in regulating hepatic GST levels. Growth hormone secretion from the pituitary gland differs markedly between the sexes, and such studies strongly suggest that testosterone regulates the hepatic expression of the Yf subunit indirectly through the male-specific pattern of growth hormone secretion (**Dolan, 1990**). Other steroid hormones have also have been found to influence GST levels, such as glucocorticoids which produce increased GST expression in both neonatal and foetal rat hepatocytes **(Mukhtar *et al.*, 1979; Sheratt *et al.*, 1989)**.

Peptide hormones also regulate GST levels. For example, thyroid hormones have been shown to decrease GST activity along with decreased levels of Ya, Yc and Yb_1 subunits **(Beckett *et al.*, 1986 and 1988)**. Other studies using thyroidectomised rats revealed an increase in both Ya and Yc subunits which were down-regulated again following subsequent treatment with thyroid hormone **(Arias *et al.*, 1976)**.

Hypophysectomised animals have been widely used in the study of hormonal control of GST expression. Experiments in the rat have revealed an increase in the levels of both hepatic and renal GSTs following hypophysectomy **(Hales & Neims, 1976; Lamartiniere, 1981)**. Hypophysectomised rats were also treated with somatotrophin **(Lamartiniere, 1981)** which had the effect of decreasing GST levels in both males and females. A similar down-regulatory effect has been shown by ACTH in the rat adrenal **(Mankowitz *et al.*, 1990)**. Hypophysectomised animals in this study showed a marked increase (i.e. 10-15-fold) in expression of the Yb_2 subunit in the adrenal gland, which could be partially suppressed following administration of ACTH. Smaller elevations of GST levels observed in the liver and ovary in these hypophysectomised animals were not affected by ACTH treatment. This apparent effect of ACTH in down-regulating GST levels in the rat adrenal has been further confirmed by studies using rat adrenocortical cells in primary culture **(Mankowitz *et al.*, 1991a)**. The significance of these findings in the context of the adrenal cortex and their relevance to this thesis will be discussed in the next section of this

chapter.

4. The Adrenal Gland

(a) Morphology and Function

In man, the two adrenal glands are small, triangular in shape, and bilaterally positioned at the superior poles of each kidney. Their total weight in humans is between 8 and 12 g, depending upon age, and they are usually heavier in females than in males. The gland consists of two morphologically and functionally distinct regions, the outer steroid-synthesising cortex, and the inner catecholamine-producing medulla. The cortex makes up 80-90% of the weight of the gland and, although the cortex and medulla come together to make up the whole adrenal gland in mammals, they are separate during the early stages of embryonic growth with the cortex arising from the mesoderm and the medulla deriving from neural crest cells of ectodermal origin (**O'Riordan *et al.*, 1985**).

Very early studies by **Brown-Sequard (1856)** showed the adrenal gland to be essential to life, although it was **Wheeler & Vincent (1917)** who showed that the ability to sustain life was due to the outer cortex and not the inner medulla. The cortex is now known to be made up of three zones, named the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR), which were first identified by **Harley (1858)**, although it was **Arnold (1866)** who actually named them. These zones are shown in the cross-section of a typical adrenal gland (Fig. 1f). The divisions were originally made on the basis of morphological differences between cells, although it is now clear that these cells also perform different functions. The ZG is the outermost zone of the cortex which adheres to the capsule and consists of round-shaped cells with relatively small cytoplasmic volume. This zone is the site of mineralocorticoid synthesis: these steroids, the most important of which is aldosterone, are involved in regulating renal Na^+ handling. The next zone inwards, the ZF, comprises the bulk of the cortex with cells arranged in columns containing abundant lipid deposits and many more mitochondria than the ZG. The ZF is largely responsible for the synthesis of glucocorticoids, the most important of which are cortisol and corticosterone.

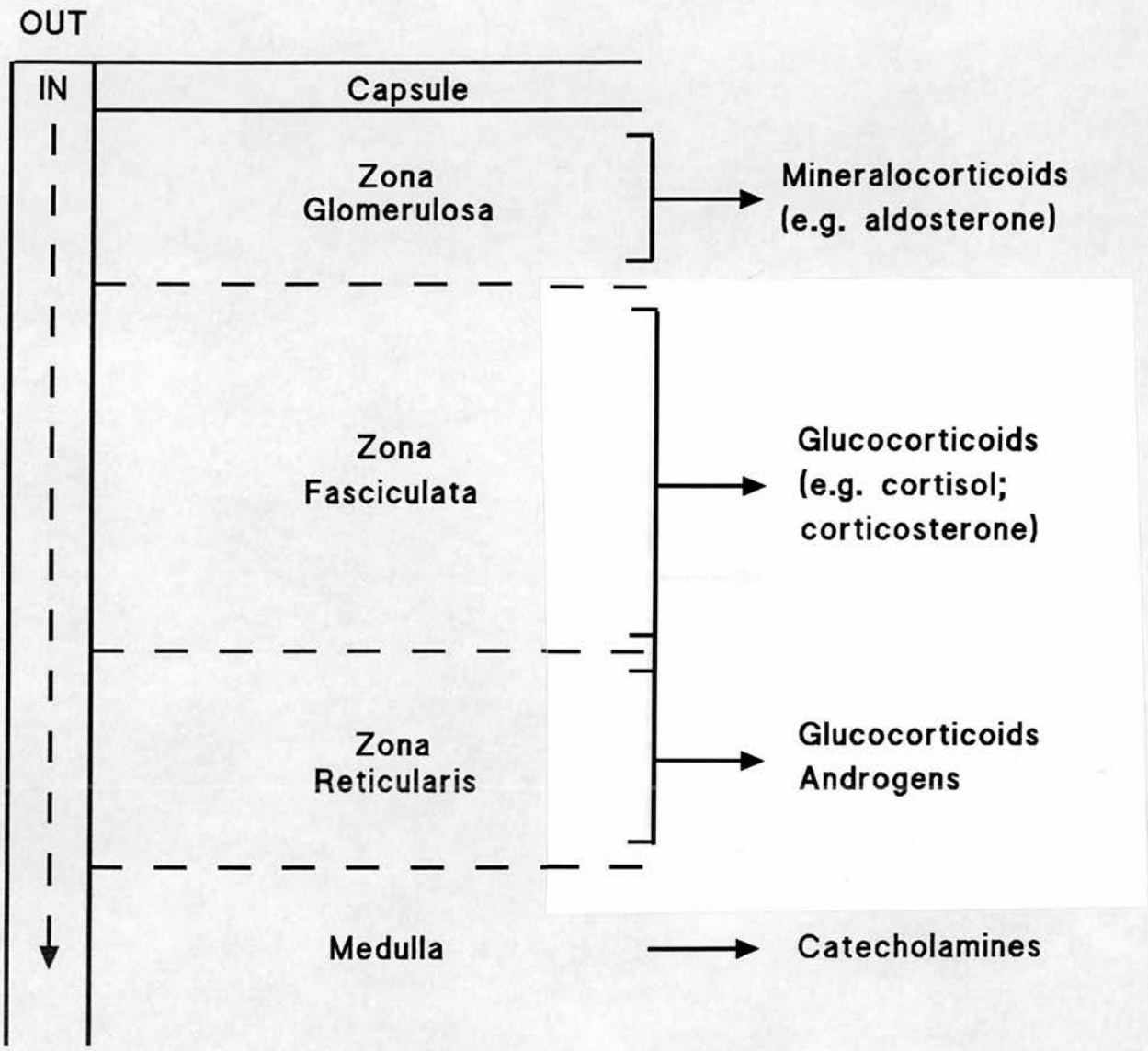


Figure 1f: Schematic Cross-Section of an Adrenal Gland.

The glucocorticoids exert important effects on carbohydrate, lipid, and protein metabolism. The ZR is the innermost zone, adjacent to the medulla, consisting of cells which resemble ZF cells although generally containing less lipid. This zone is known to produce androgens although in relatively low amounts as compared to the gonads. The daily production rate of androgens by the adrenal cortex, as well as the other adrenocortical steroids, is shown in Table 1e for a normal human adult.

An interesting observation concerning the adrenal gland is that differences exist between male and female rat adrenal glands at both the structural and functional level. For example, the weight of the adrenal gland and the volume of cells in the ZF/ZR are greater in female rats **(Malendowicz *et al.*, 1986)**, as is the level of pregnenolone synthesis and corticosterone secretion **(Kitay, 1961)**. Recent investigations have shown that there is no significant difference in plasma ACTH levels in male and female rats, although human females have more pulses of plasma ACTH with higher amplitudes than do males **(Horrocks *et al.*, 1990)**. Such observations, along with many more conflicting results, suggest the influence of the pituitary-hypothalamus axis on sex differences in the adrenal to be highly complex, and as yet not fully understood.

4.(b) Steroid Biosynthetic Pathway

The enzymes required for the synthesis of steroids in the adrenal cortex are present both in the inner mitochondrial membrane and in the endoplasmic reticulum (**O'Riordan *et al.*, 1985**). The precursor of all steroid hormones is cholesterol, with about 80% of cholesterol in the adrenal being taken up from the blood, where it is transported by lipoproteins such as LDL and HDL **(Toth *et al.*, 1986)**. Once it reaches the cytoplasm of adrenocortical cells, the cholesterol is stored in lipid droplets in the form of cholesterol esters **(Boggaram *et al.*, 1984 and 1985)**. In response to ACTH stimulation, cholesterol esters are hydrolysed and the cholesterol transported to the mitochondria where the first key step of steroid synthesis occurs (see Fig. 1g for this, and all of the following, reactions). The transport of cholesterol to the mitochondria is now thought to involve a sterol carrier protein **(Vahouny *et al.*, 1984 and 1985)**, although there is also evidence for the involvement of microfilaments in its transport, since their functional impairment causes

Steroid	Rate
Cortisol	100
Corticosterone	5
Aldosterone	0.75
11–Deoxy–cortisol	3
Deoxy–corticosterone	1.2
Androstenedione	3
Dehydroepiandrosterone Sulphate	125
Dehydroepiandrosterone	8
Testosterone	0.06
Oestrone	0.02

Table 1e: Daily Human Adult Adrenal Steroid Production Rate Relative to Cortisol.

(normal adult human cortisol production rate = 18–30 mg/day)
(Neville & O'Hare, 1982, chapter 8)

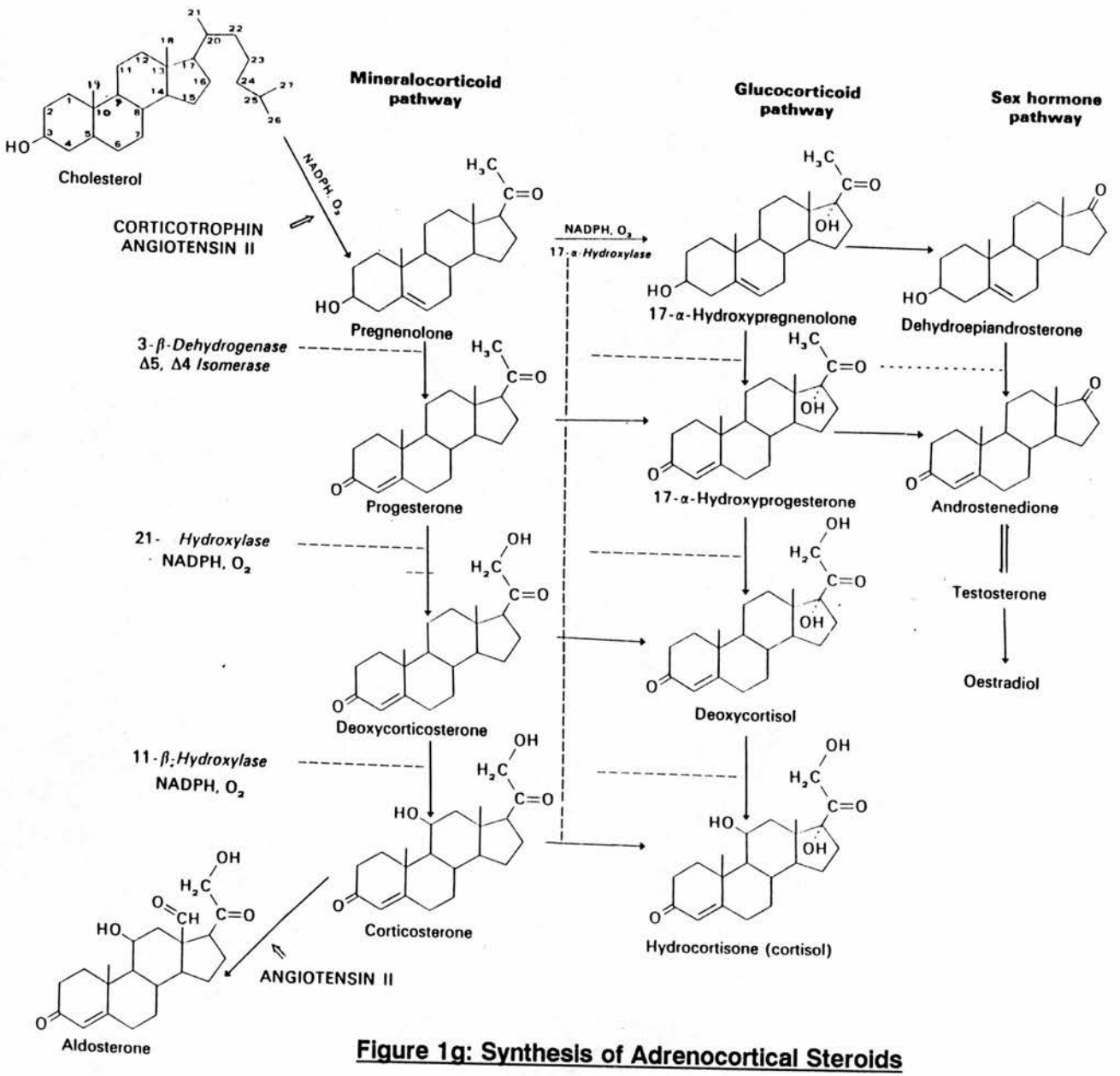


Figure 1g: Synthesis of Adrenocortical Steroids

(from **Bowman & Rand, 1984, chapter 19**)

inhibition of both ACTH and cAMP responsiveness **(Hall, 1984)**.

The first reaction of steroid synthesis in the mitochondria is the cleavage of cholesterol to form pregnenolone. This reaction is the rate-determining step in steroid formation, and is catalysed by the cholesterol side-chain cleavage enzyme which is a cytochrome P450 ($P450_{scc}$) **[Jefcoate *et al.*, 1986]**. The requirement for a cytochrome P450 enzyme is a feature of several enzymatic steps during steroidogenesis. Pregnenolone, formed by $P450_{scc}$ activity, is then transferred across the mitochondrion to the endoplasmic reticulum where (in humans, sheep, cattle and pigs) another cytochrome P450 enzyme, $P450_{17\alpha}$, converts pregnenolone to 17-α-hydroxypregnenolone. Importantly, in some species (such as the rat) this enzyme is lacking. The next step is carried out by an enzyme complex displaying both 3-β-hydroxysteroid dehydrogenase and $\Delta^{5,4}$isomerase activities, which can convert 17-α-hydroxypregnenolone to 17-α-hydroxyprogesterone. The same enzyme can also act on pregnenolone, converting this substrate to progesterone. Cytochrome $P450_{C21}$ then converts these products to the 11-deoxy versions of either corticosterone (from progesterone) or cortisol (from 17-α-hydroxyprogesterone). These are subsequently transported back to the mitochondria where the final reaction involving cytochrome $P450_{11\beta}$ produces either cortisol or corticosterone, respectively.

The relative amounts of each final steroid product in adrenocortical cells are thought to be regulated by the relative amounts of steroid-synthesising enzymes present in each pathway **(Jefcoate *et al.*, 1986)**. Importantly, corticosterone is converted to aldosterone in the ZG of all species by the action of an 18-hydroxylase. Also, in species containing a cytochrome $P450_{17\alpha}$ enzyme, the lyase activity associated with this enzyme enables the further formation of both dehydroepiandrosterone and androstenedione, both of which serve as precursors for sex hormones in the adrenal cortex and other tissues.

An important feature of steroid synthesis is that the expression of the different steroid synthesising enzymes is both species- and tissue-specific. For example, as mentioned above, only certain species express the cytochrome $P450_{17\alpha}$ enzyme which is necessary for cortisol production. Other species (including rats, mice, hamsters and rabbits) do not express this enzyme and consequently produce corticosterone. In terms of tissue-specificity, adrenocortical cells are

the only ones expressing cytochromes $P450_{11\beta}$ and $P450_{C21}$, and therefore are the only cells which can produce glucocorticoids.

4(c) Hormonal Control of Adrenocortical Steroidogenesis

(i) By ACTH

Regulation of ACTH Secretion

The anterior pituitary at the base of the brain releases several peptide hormones, including adrenocorticotropin (ACTH), which have different effects on their target cells. The actions of ACTH are largely confined to the adrenal cortex where it stimulates cortisol synthesis by increasing the conversion of cholesterol to pregnenolone in ZF and ZR cells as described above **(Simpson & Waterman, 1988)**. Control of ACTH secretion is, in turn, thought to be largely under the influence of corticotropin-releasing hormone (CRH) released by the hypothalamus (Fig. 1h). The secretion of CRH is determined by blood cortisol levels, which exert a negative feedback effect on hypothalamic CRH-containing neurones during high levels of secretion. Cortisol also acts directly on pituitary corticotrophs, inhibiting ACTH secretion when levels of cortisol in the blood are increased. A schematic summary of the negative control of cortisol secretion in the adrenal cortex is shown in Fig. 1h. One of the most characteristic features of ACTH secretion is its circadium rhythm, which is related to light and dark cycles: ACTH concentration is lowest around midnight and increases until the time of rising, slowly declining thereafter. ACTH release is also episodic and can be stimulated by stresses such as pain, fear, fever or hypoglycaemia (**O'Riordan *et al.*, 1985**).

Mechanism of action of ACTH

Hormones are known to exert their effects on target cells by binding to specific receptors which are located on the surface of the cell. Such binding triggers the activation of intracellular messenger systems which then mediate the effect(s) of the hormone (**O'Riordan *et al.*, 1985**). One of the most extensively studied intracellular messenger systems is the one involving cAMP **(Grahame-Smith *et al.*, 1967)**. Following ACTH binding to its receptor, activation of a plasma membrane-bound enzyme complex, adenylate cyclase, leads to the synthesis of cAMP from ATP

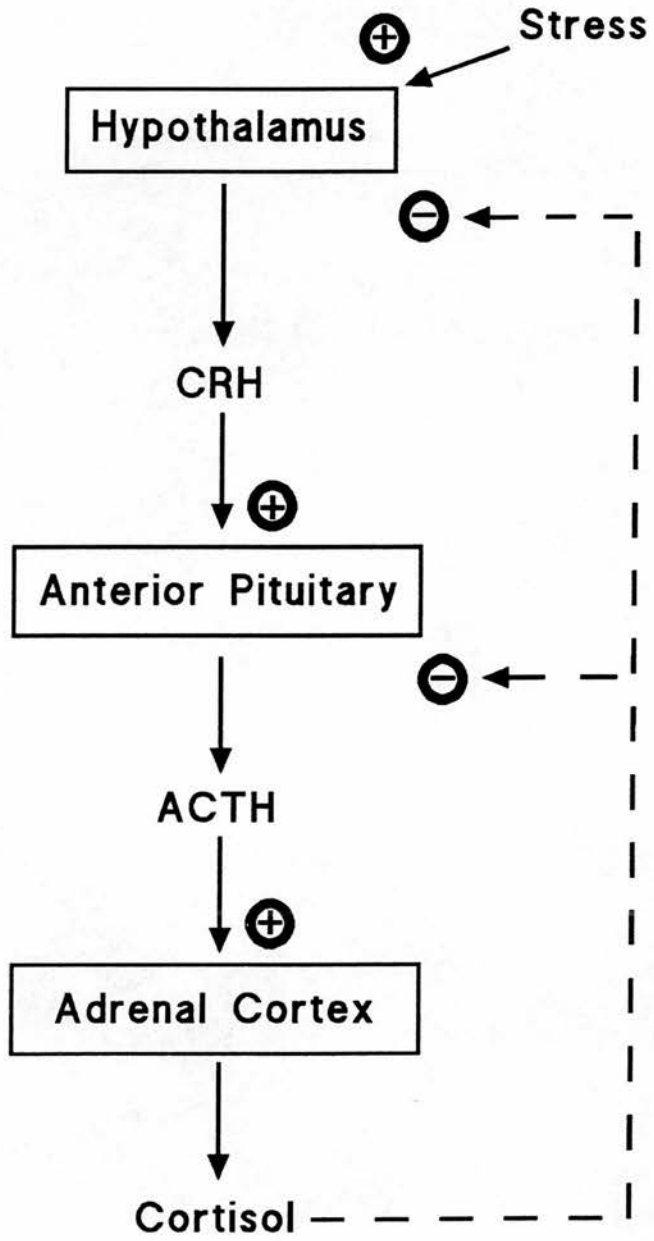


Figure 1h: ACTH Control of Cortisol Production from the Adrenal Cortex.

(adapted from O'Riordan, Malan & Gould, 1985, chapter 2)

in the cell. In many cases the hormone receptor is known to be linked to the enzymatically active cyclase unit via a GTP binding protein ("G" protein) which acts as a regulator of hormone action by slowly hydrolysing GTP (**see Stryer & Bourne, 1986; for a review, Ross, 1989**). There is now known to be a large family of G proteins mediating the effects of many agonists, of which three have been most characterised: G_s, which mediates hormonal stimulation of adenylate cyclase; G_i, which mediates hormonal inhibition of adenylate cyclase; and G_o, which mediates hormonal activation of phospholipase C (see later).

The cAMP released inside the cell is then known to activate a protein kinase(s) (protein kinase A) which phosphorylates other proteins, thereby regulating their activity. In the case of ACTH, the reaction cascade eventually ends in the phosphorylation (and subsequent activation) of cholesterol-ester hydrolase, which cleaves fatty acids from cholesterol in the lipid droplets of adrenocortical cells, leaving free cholesterol available for further metabolism **(Tait *et al.*, 1980)**. Cyclic AMP, through activation of protein kinase A, also brings about transcriptional activation of certain key steroidogenic enzymes **(Simpson & Waterman, 1988)**.

Application of molecular biology to intracellular signalling

The advent of molecular biology technology has enabled the finer details of this intracellular signalling system to be established. Experiments investigating cAMP-mediated induction of genes have revealed two distinct mechanisms of action: one is rapid (occurring within minutes) and does not involve protein synthesis, and the other takes much longer to occur (i.e. hours) and is thought to involve protein synthesis (for a review, see **Roesler *et al.*, 1988**). Further experiments have shown that several genes regulated by cAMP contain a consensus sequence, or CRE (cAMP regulatory element), responsible for both types of induction **(Montminy *et al.*, 1986)**. Another sequence has been reported to be regulated by cAMP, called the transcription factor AP-2 regulatory site, which is also activated by phorbol esters in addition to cAMP **(Imagawa *et al.*, 1987).** Some work has also concentrated on identifying the actual proteins which bind to cAMP regulatory sites, and indeed some have been discovered such as CREB - cAMP responsive element binding protein **(Montminy *et al.*, 1987)**.

Molecular biological techniques have also been specifically applied to understanding the

mechanisms which regulate steroid metabolising enzymes. For example, increases in mRNA levels corresponding to all the steroid hydroxylases in response to either cAMP or ACTH have been found **(Zuber *et al.*, 1986; Di Blasio *et al.*, 1987)**, in addition to the results of both nuclear run-on experiments **(John *et al.*, 1986, a; b)** ,where it is possible to measure rates of transcription, and studies of mRNA stability **(Boggaram *et al.*, 1989)**. These experiments have collectively shown an increase in mRNA due to transcriptional activation of the corresponding genes along with an increase in mRNA stability, especially with the cytocrome $P450_{scc}$ enzyme. The actual mechanism of induction of various steroid hydroxylases by cAMP is believed to involve labile proteins called steroid hydroxylase-inducing proteins, or SHIP **(John *et al.*, 1986, a; b)**, although information on these proteins is limited at present.

It would appear that cAMP-independent regulation of steroid hydroxylases also occurs since unstimulated bovine adrenocortical cells, in the absence of ACTH, are able to maintain a basal level of both steroid-synthesising enzymes and steroid secretion **(Waterman & Simpson, 1989)**. Also, it has been proposed in the rat that ACTH may act via an alternative phospholipase C-mediated intracellular signalling system (see below) to promote steroidogenesis **(Whitley *et al.*, 1987)**. However, in the bovine adrenal cortex there is no reported evidence of low-dose effects of ACTH on phospholipid turnover in either ZG, ZF or ZR cells.

4.(c)

(ii) By Angiotensin II (AII)

Regulation of Secretion

Angiotensin II (AII) is believed to be the main stimulant of aldosterone production from ZG cells of all species **(Kaplan & Bartter, 1962)** in a process involving the proteolytic enzyme, renin. This enzyme, which is synthesised in the juxtaglomerular cells surrounding the afferent arteriole in the glomeruli of the kidney, uniquely splits a leucine-leucine peptide bond of a circulating α_2-globulin called angiotensinogen. Under the influence of renin, a decapeptide is split off angiotensinogen called angiotensin I. This latter peptide is largely biologically inactive, but is converted in several tissues (especially the lung) into angiotensin II. Normally, in man,

angiotensinogen is present in adequate concentrations, and the rate-limiting step for AII production is the concentration of renin. One of the main factors controlling renin secretion is sodium flux across the macula densa of the distal tubule in the kidney. When the flux is high the rate of secretion is suppressed, lowering AII concentrations and consequently aldosterone secretion.

Mechanism of Action

In rat ZG cells AII is known to promote a rise in intracellular Ca^{2+} ion concentration, which is consistent with the activation of phospholipase C. Phospholipase C activation by agonists is now a well-characterised mechanism of signal transduction for a number of hormones and neurotransmitters (as reviewed by **Berridge, 1984)**, and a number of agonists which utilise this signal transduction mechanism have been found to stimulate adrenocortical steroidogenesis (for a review, see **Bird *et al.*, 1990).** Hormonal activation of phospholipase C causes the breakdown of inositol-containing membrane phospholipids to inositol phosphates and diacylgycerol. These latter compounds exert different effects within the cell: inositol-1,4,5-trisphosphate, derived from the breakdown of phosphatidylinositol 4,5-bisphosphate, is known to bring about the release of Ca^{2+} ions from non-mitochondrial intracellular store, whereas diacylglycerol stimulates protein kinase C which is a multiprotein kinase. AII stimulation of phospholipase C in ZG cells is known to require a G protein which links the activation of the receptor to phospholipase C (**Baukal *et al.*, 1988**). There is also evidence to suggest that AII links to a G_i protein in ZG cells, leading to inhibition of adenylate cyclase.

Presently, there appears to be very little knowledge of the actual mechanism by which activation of phospholipase C stimulates steroidogenesis, although it seems possible that the corresponding release of intracellular calcium has an effect of activating a chain of phosphorylation steps by causing the initial activation of a calmodulin-dependent protein kinase. This enzyme would then finally activate the steroid synthesising enzymes at both the early and late stages of the pathway **(Quinn & Williams, 1988).**

In both the bovine and rat ZF/ZR cells, AII is known to produce an increase in phospholipase C-mediated inositol phosphate production, although only in bovine adrenocortical

cells is there a corresponding increase in steroid output **(Whitley *et al.*, 1987; Bird *et al.*, 1989)**. Further experiments by **Bird *et al* (1989)** have revealed that, in bovine ZF/ZR cells, phospholipase C is the sole mediator of All-stimulated cortisol production.

3 (C)

(ii) by other effectors

Bovine ZF/ZR cells have been shown to respond steroidogenically to both adrenergic and cholinergic agonists **(Hadjian *et al.*, 1981, 1982, 1983; Kawamura *et al.*, 1984, 1985)**. Such findings have important implications since they open up the possibility that, in addition to endocrine control of steroidogenesis (e.g. by All or ACTH)I, there may be also an innervative control of adrenocortical steroidogenesis and possible paracrine control. Experiments using primary cultures of bovine adrenocortical cells have shown (in the bovine system at least) catecholamines to stimulate cortisol secretion from these cells via a cAMP-dependent mechanism **(Walker *et al.*, 1988)**, and that β_1-adrenoreceptors are responsible for mediating this effect **(Lightly *et al.*, 1990)**. **Hadjian *et al* (1981, 1982)** showed that freshly-isolated bovine ZF/ZR cells were steroidogenically responsive to acetylcholine which acted via muscarinic receptors. Experiments investigating the cholinergic response in primary cultures of bovine adrenocortical cells have revealed that acetylcholine stimulates cortisol secretion via the M3-type muscarinic receptor, which is linked to a phosphoinositide-specific phospholipase C **(Walker *et al.*, 1990)**.

Hinson *et al* (1987) were the first to demonstrate that both aldosterone and corticosterone secretion were stimulated in a dose-dependent manner by vasopressin using both the perfused rat adrenal gland and isolated superfused cells. Experiments using cultured bovine ZF/ZR cells have shown vasopressin to stimulate a small though significant increase in cortisol secretion with a corresponding increase in phosphoinositol formation **(Bird *et al.*, 1990)**.

Experiments have shown that an increase in the concentration of K^+ ions in the plasma leads to an increase in aldosterone production from ZG cells **(Tait *et al.*, 1980)**. The current theory is that K^+ ions are responsible for opening voltage-gated Ca^{2+}-channels in the plasma membrane **(Quinn & Williams, 1988)**, and thus extracellular K^+ ions cause an increase in intracellular Ca^{2+} ion concentration. Both serotonin and dopamine have also been shown to have

effects on adrenocortical steroidogenesis. In the rat, for example, serotonin stimulates aldosterone production from ZG cells via a cAMP-dependent mechanism **(Muller & Ziegler, 1968)**. On the other hand, dopamine has been shown to inhibit AII-stimulated aldosterone production from bovine ZG cells **(Racz *et al.*, 1984)**, although has no effect on ACTH-stimulated aldosterone production **(Connell *et al.*, 1986)**. Presently, there is no reported evidence that K^+ ions, serotonin or dopamine have any effect on steroidogenesis in bovine ZF/ZR cells.

5. Toxicity in the Adrenal Cortex

(a) Metabolism of Xenobiotics and Toxicity

Several studies have shown the adrenal cortex to display a high capacity to accumulate hydrophobic and potentially toxic xenobiotics such as polyhalogenated biphenyls **(Castracane *et al.*, 1982; Mohammed *et al.*, 1985; Lund *et al.*, 1988)**. Such accumulation can be attributed to the high degree of hydrophobicity of these compounds and their solubility in the large amount of lipid droplets in adrenocortical cells (the intracellular stores for cholesterol). However, the adrenal cortex not only accumulates hydrophobic xenobiotics but is also known to be highly and specifically vulnerable to many such compounds, which include dimethylbenz[a]anthracene (DMBA), carbon tetrachloride, acrylonitrile and 1-chloro-2-[2,2-dichloro-1-(4-dichlorophenyl)ethyl]benzene **(Hornsby, 1989)**. Other polycyclic aromatic compounds are known which do not cause adrenal necrosis such as benz[a]anthracene, benzo[a]pyrene and 3-methylcholanthrene.

Adrenocortical cell damage in the rat following DMBA administration appears to require both the metabolism of DMBA to a more toxic form by the liver, and also the presence of specific cytochrome P450 species in the adrenal cortex **(Hallberg, 1990)**. Some experimental evidence for this comes from studies involving liver damage by carbon tetrachloride prior to DMBA administration, in which DMBA does not exert the same toxic effect **(Wheatly *et al.*, 1966)**. DMBA is metabolised by cytochrome P450 species which also have aryl hydrocarbon hydroxylase activity

(Hallberg, 1990), with the subsequent generation of toxic oxygen metabolites, thus leading to oxidative damage **(Hallberg & Rydstrom, 1987)**. Evidence for the existence of oxidataive mechanisms being involved in DMBA toxicity in the adrenal cortex comes from experiments in which the administration of the antioxidants BHA or ethoxyquin reduced this toxicity greatly **(Wattenberg,1972)**.

An interesting feature of DMBA-induced adrenotoxicity is the requirement for ACTH **(Hallberg *et al.*, 1983)**, and efforts have been made to correlate the activities of ACTH-dependent steroid hydroxylases and the toxicity of DMBA **(Hallberg *et al.*, 1983; Hallberg & Rydstrom, 1987)**. However, the results from such studies have proved to be inconclusive, and the exact manner in which ACTH is involved in this toxicity is still unclear.

Several attempts have been made to induce glutathione S-transferases in the rat adrenal cortex using a number of classic xenobiotic inducers of hepatic GSTs in the rat and mouse, such as 3-methylcholanthrene and phenobarbital **(Bengtsson *et al.*, 1983; De Pierre *et al.*, 1984).** Results from such experiments have proved inconclusive, and in the majority of such studies little or no effect has been observed. The reason for this apparent lack of inducibility is unclear, although the observation that the rat adrenal cortex lacks a functional Ah receptor **(Carlstedt-Duke, 1979)** may provide part of the answer.

5. (b) Glutathione S-transferases in the Adrenal Cortex

A number of studies have been carried out to determine GST levels in the adrenal gland, usually as part of a general species-specific organ survey of GST activity. The adrenal glands from several species have been used in such studies, including the rat **(Sierakowski & Kraus, 1984; Gay & Ehrich, 1990)**, human **(Sherman *et al.*, 1983; Pacifici *et al.*, 1986; Faulder *et al.*, 1987; Corrigall & Kirsch, 1988)**, bovine **(Aceto *et al.*, 1986; Hayes *et al.*, 1989b)**, and even the chicken **(Gay & Ehrich, 1990)**. Many of these studies (especially the rat) have been limited by the use of the whole adrenal gland. Conclusive data concerning the adrenal cortex can clearly only be obtained following separation of the cortex from the medulla. **Hayes *et al* (1989b)** manually dissected the cortex from the medulla, and were able to show that the cortex displayed

approximately 3-fold more GST activity than the medulla.

Much of the previous work investigating GST levels in the adrenal gland has also concentrated on studies of whole tissue cytosolic fractions, with very little emphasis on the actual isoenzyme distribution. However, a limited analysis of the GST isoenzyme complement of the rat adrenal gland using chromatofocusing has identified a major subunit which has a basic isoelectric point, with several other minor proteins with both near-neutral and acidic isoelectric points **(Sierakowski & Kraus, 1984)**. **Ostlund Farrants *et al* (1987)** used reverse-phase h.p.l.c. to demonstrate that the major isoenzyme in the rat adrenal gland was composed of basic Yc subunits. This technique further showed the presence of low amounts of other subunits, with the Yb_1 subunit being the most abundant of these proteins; the Ya subunit was almost undetectable. In contrast, **Hayes *et al* (1989b)** have shown the bovine adrenal cortex to contain relatively high levels of a Ya-type subunit, with very small amounts of Yc-type subunits. This investigation of GST expression in different bovine organs, using antibodies raised against rat GST isoenzymes, also revealed a subunit distribution in the adrenal medulla which differed from that in the cortex. This again illustrates the caution which is required in the interpretation of data obtained from the whole adrenal gland and the requirement to separate the cortex from the medulla.

An interesting observation concerning GSTs in the adrenal gland has been the discovery that the theta-class GST has been found at relatively high levels in this organ **(Hiratsuka *et al*., 1990)**. Although initially isolated from rat liver, this enzyme has subsequently been found in several other organs, notably the adrenal cortex, where the activity is only exceeded by the liver and testes.

As with adrenal glands from the rat (containing largely the Yc subunit) and bovine (containing largely the Ya subunit) species, both human adult and foetal adrenal cortex tissues were found to express a basic subunit **(Pacifici *et al*., 1986; Faulder *et al*., 1987; Corrigall & Kirsch, 1988)**. This would imply a specific function for basic GSTs in the normal functioning of the adrenal cortex. The importance of GSTs in this organ is given further support from the work of **Hayes *et al* (1989b)** which revealed that, of all bovine organs studied, GST activity in the adrenal cortex was exceeded only by the liver.

One possible function for GST in the adrenal cortex is in endogenous detoxification. The synthesis of steroid hormones by the adrenal cortex requires the use of molecular oxygen, with a corresponding problem of toxicity (especially membrane damage) caused by lipid peroxidation **(Hornsby & Crivello, 1983a)**. That lipid peroxidation and other aspects of oxygen toxicity are a potential problem for this tissue is reflected in the relatively high levels of biological antioxidants (e.g. superoxide dismutase, ascorbic acid, α-tocopherol and glutathione) which presumably limit the damage caused by such oxidative stress **(Hornsby & Crivello, 1983b)**. Evidence now suggests that the GSTs may also be involved in combatting this oxidative stress prevailing during steroid biosynthesis, since several of the compounds generated in the process of peroxidation have been found to act as substrates for GST. These include 4-hydroxyalkenals **(Ålin *et al.*, 1985)**, cholesterol-α-oxide **(Meyer & Ketterer, 1982)** and DNA hydroperoxides **(Ketterer *et al.*, 1987)**. Experiments with the 4-hydroxyalkenals, in particular, have shown these compounds to be produced in relatively large amounts during stimulated lipid peroxidation **(Esterbauer *et al.*, 1982; Poli *et al.*, 1985)**. Other investigations have shown that alpha-class GSTs, in addition to the selenium-dependent form of glutathione peroxidase, are able to combat lipid peroxidation *in vitro* **(Tan *et al.*, 1984)**. This peroxidase activity of alpha-class GSTs (described earlier in this chapter) may limit endogenous toxicity in the adrenal cortex as a consequence of the relatively large production of potentially harmful hydroperoxides and various free oxygen radicals formed as bi-products during the synthesis of steroids. The relatively high levels of glutathione (and selenium) observed in the adrenal cortex **(Hornsby & Crivello, 1983b)** give further support for an *in vivo* function for this peroxidase activity, in addition to the ability of the enzyme to conjugate other potentially toxic species such as the 4-hydroxyalkenals.

Experiments have been carried out very recently to investigate the possibility that GSTs expressed in the rat adrenal gland are under hormonal influence **(Mankowitz *et al.*, 1990)**. Results here have shown that, following hypophysectomy, the Yb_2 subunit shows a 15-fold elevation in expression, an effect which can be partially overcome by the administration of ACTH to the hypophysectomised animals. A similar phenomenon has also been seen in rat adrenocortical cells in primary culture, where prolonged culture results in the elevated expression

of the same Yb_2 subunit, which can again be partially overcome by ACTH (or cAMP) **[Mankowitz *et al.*, 1991a]**. There would, therefore, appear to be an inhibitory effect of ACTH on the expression of the Yb_2 subunit in rat adrenal cortex. This apparent influence of ACTH on GST expression is further complicated by sex differences, whereby male and female rats appear to show both differences in the GST subunits affected by ACTH and the actual magnitude of these changes in GST subunit expression **(Mankowitz *et al.*, 1991b)**.

4. Aims of the Thesis

(1) To fully characterise the GST isoenzymes expressed by different bovine organs.

(2) To study in detail the GST subunit composition of the cytosolic fraction from bovine adrenal cortex.

(3) To use the information obtained in (2) to study the expression of GSTs in primary cultures of bovine adrenocortical cells and their possible regulation of expression by steroidogenic agonists.

(4) In the longer term, for the work to serve as a basis for determining the precise function of GSTs in the adrenal cortex, bearing in mind the steroidogenic nature of this organ and the consequental problems for adrenocortical cells resulting from steroid production.

Chapter 2: Materials and Methods.

2.01 MATERIALS

(a) Chemicals

Commercially available materials were obtained from the following sources:-

Aldrich Chemical Co. Ltd., New Road, Gillingham, Dorset, SP8 4JL

trans-4-phenyl-3-buten-2-one.

BDH Chemicals Ltd., Burnfield Avenue, Thornliebank, Glasgow G46 7TP

Acetic acid; acrylamide; bromophenol blue; butan-2-ol; disodium hydrogen orthophosphate; ethanol; glycerol; glycine; hydrochloric acid; hydrogen peroxide; 2-mercaptoethanol; methanol; NN'-methylenebisacrylamide; orthophosphoric acid; sodium chloride; sodium dodecyl sulphate; sulphuric acid; trichloroacetic acid; urea.

Bio-Rad Laboratories, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Herts HP2 7TD

4-chloro-1-naphthol; goat anti (rabbit IgG)-HRP second antibody conjugate; 2-mercaptoethanol; NNN' N'-tetramethyl ethylenediamine (TEMED).

CIBA Laboratories, Horsham, West Sussex, England

Adrenocorticotropic hormone ($ACTH_{1-24}$)

Elkay Labsystems Group (UK) Ltd., Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hants RG21 2YH

Pump tubing.

Henry Simon Ltd., P O Box 31, Stockport, Cheshire SK3 0RT

Nylon gauzes (250, 100 and 30 μm).

ICN Biomedicals Ltd., Lincoln Road, Cressex Industrial Estate, High Wycombe, Bucks HP12 4XJ

Reagent grade bovine serum albumin (fraction V).

Koch Light Laboratories, Colinbrook, Berks, U.K.

Cumene hydroperoxide.

LKB Products, Broma, Sweden

Ampholine solutions.

Lorne Diagnostics, P O Box 6, Tywford, Reading, Berks RG10 9NL

Collagenase (type 1).

Millipore (UK) Ltd., Harrow, Middlesex, England

Ultrafiltration membranes (0.45 μm).

MRC Division of Biological Standards, National Institute for Medical Research, Mill Hill, London NW7 1AA

Angiotensin II (asp^1-val^5).

Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, Northumberland NE23 9HL

Amphotericin B; Costar cell culture flasks (75 cm^3); Earle's balanced salt solution; Ham's F10 growth medium; penicillin; streptomycin; trypsin-EDTA (0.05% stock solution).

Pharmacia Fine Chemicals, Uppsala, Sweden

Protein standards for isoelectric focussing.

Rathburn Chemicals, Walkerburn, Peeblesshire, Scotland

Acetonitrile (h.p.l.c. grade); trifluoroacetic acid (gas sequence analyser grade).

Schleicher and Schuell, P O Box 4, Dassel, West Germany

Nitrocellulose.

Scientific Instrument Centre Ltd., London, U.K.

Visking dialysis tubing (molecular cut-off approx. Mr 10 000).

Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH

Ammonium persulphate; controlled process serum replacement (CPSR-5); 1-chloro-2,4-dinitrobenzene; Coomassie Brilliant Blue R-250; 1,2-dichloro-4-nitrobenzene; dithiothreitol; ethacrynic acid; ethanolamine; Freund's adjuvant (complete and incomplete); gelatin; glutathione (reduced form); glutathione reductase; glutathione-Sepharose 6B; glycine; nicotinamide adenine dinucleotide phosphate (type X); p-nitrobenzyl chloride; p-nitrophenyl acetate; phosphate buffered saline (PBS) tablets; polyoxyethylene-sorbitan monolaurate (Tween 20); Sephadex G10; Sephadex G50; sodium azide; sodium chloride; Trizma Base.

2.01

(b) Materials Obtained from Non-commercial obtained Sources

Antisera and Protein Standards

GST isoenzyme mixtures from rat liver cytosol (Ya, Yb and Yc subunits of Mr 25 500, 26 300 and 27 500, respectively) were used as molecular weight standards during SDS/PAGE, and were prepared by Dr J D Hayes, as were the human alpha, mu and pi class GST standards. Antisera raised against individual rat GST subunits, also supplied by Dr J D Hayes, were used during initial immunoblotting of bovine adrenal cortex GST. The specificities of these antibodies have been previously described by **Hayes & Mantle (1986b)**.

During this thesis antisera were raised against several of the bovine GST subunits. Portions of purified GST (approximately 0.25 mg/ml) were separately emulsfied with an equal volume of Freund's complete adjuvant. Between 100 and 200 μg of each emulsion was injected subcutaneously at several distinct sites on the back of female New Zealand white rabbits. After six weeks, each rabbit was re-inoculated with approximately 100 μg of the original immunogen in Freund's incomplete adjuvant, which was repeated after a further two weeks (i.e. on the 8th week after initial inoculation). On the 10th week, the animals were killed and bled. The blood was left to clot at 4°C overnight, and the next day the serum removed. Finally, the serum was centrifuged (450 x g; 15 min) to remove red blood cells and then stored at -80°C.

Chemicals

The Δ^5-androstene-3,17-dione was a gift from Dr P K Stockman (Department of Clinical Chemistry, The Royal Infirmary, Edinburgh), as was the 4-hydroxynonenal which was given by Prof. Hermann Esterbauer (Department of Biochemistry, University of Graz, Schubertstrasse, A-8010 Graz, Austria).

S-hexylglutathione was synthesised by coupling iodohexane to reduced glutathione via the cysteine sulphydryl group as described by **Vince *et al* (1971)**.

The S-hexylglutathione-Sepharose 6B affinity matrix was made by coupling the γ-glutamyl

moiety of S-hexylglutathione to epoxy-activated Sepharose 6B as described by **Mannervik & Guthenberg (1981)**.

Tissue

All bovine organs were obtained from freshly-slaughtered cattle (between 18 and 24 months old) at Gorgie Abattoir (Edinburgh District Council, 2 Newmarket Road, Edinburgh EH14 1RH), collected into ice-cold saline (0.9% w/v) and transported on ice to the laboratory immediately. On arrival, all organs were roughly trimmed to remove excess fat and stored at -80°C until further use. In the case of the adrenal glands dissection was carried out according to the experimental procedure being followed: during analysis of GST isoenzymes in adrenal cortex tissue, the cortex was manually dissected from the medulla and the separated tissue stored at -80°C until use; during the preparation of primary cultures, the whole adrenal gland was trimmed of excess fat and subsequently transferred to a class II laminar flow-cabinet and sectioned as described later.

The human adrenocortical tissue used in this thesis was the right adrenal gland from a patient with bilateral adrenal hyperplasia undergoing adrenalectomy for Cushing's disease. The tissue was obtained immediately after surgery and stored at -80°C until use.

2.02 METHODS

(a) General Analytical Techniques

Protein Determination

Monitoring of the protein content in fractions eluted by the various chromatographic procedures was carried out by measuring the absorbance values spectrophotometrically at 280 nm (Uvikon Kontran 860 spectrophotometer). All other protein determinations were by the dye-binding method of **Bradford (1976)** which had been automated on a Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts, U.K.) using bovine serum albumin as

a calibration standard.

GST Enzymatic Assays

All enzyme assays were carried out in duplicate or triplicate at 37°C and non-enzymic rates determined for all substrates and subtracted from the rates observed in enzyme-containing samples. GST activities with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate were carried out using a similar method to the one described by **Habig & Jakoby (1981)**. Glutathione peroxidase activity using both cumene hydroperoxide (for the selenium-independent enzyme) and hydrogen peroxide (for the selenium-dependent enzyme) as substrates were carried out using a coupled reaction based on the method described by **Wendel (1979)**. All assays using either CDNB, cumene hydroperoxide or hydrogen peroxide were performed on a Cobas Fara centrifugal analyser. Using this instrument, a maximum of 29 samples could be analysed simultaneously for enzyme activity. In the case of GST activity with CDNB, samples were pre-incubated with GSH prior to initiation of the reaction by centrifugation of CDNB into the reaction mixture. The resulting conjugation of CDNB with GSH was measured directly by monitoring absorbance at 340 nm. A series of 8 absorbance readings were automatically taken to allow calculation of the rate of conjugation, the first at 10 seconds after adding CDNB, followed by 7 subsequent readings at 5 second intervals thereafter.

The assay using cumene hydroperoxide (CuOOH) gave a value for total glutathione peroxidase activity in a sample (i.e. both the selenium dependent and independent enzyme activities). The selenium-dependent glutathione peroxidase was specifically assayed using hydrogen peroxide (H_2O_2) and thus the difference in peroxidase activity using CuOOH and H_2O_2 gives an approximate measure of the selenium-independent glutathione peroxidase activity contributed by GST (see chapter 1). Both peroxidase assays involve a coupled reaction in which NADP formation, measured as the fall in absorbance at 340 nm as NADPH is oxidised to NADP, is used as an indicator of peroxidase activity:

$ROOH + 2\ GSH \longrightarrow H_2O + ROH + GSSG$ **Peroxidase Reaction**

$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP$ **Reductase Reaction**

By pre-incubating samples with an initial reagent containing GSH, NADPH and GSH reductase (final concentration 1 unit/μl), addition of either CuOOH or H_2O_2 initiates the reaction sequence. Ten absorbance readings were taken in both assays: using H_2O_2, the absorbance was monitored 5 seconds after the initiation of the reaction, and at 5 second intervals thereafter for a further 9 readings; for the CuOOH assay, absorbance was monitored 5 seconds after initiation of the reaction , with measurements being similarly taken but at 10 second intervals.

In order to convert rates of reaction into appropriate units of enzyme activity, linear regression analysis was performed using an in-built kinetic rate programme for those assays carried out on the Cobas Fara. Using a pre-programmed conversion factor, values for the CDNB assay were presented as ΔA/min/ml, which could then be converted to units of μmol/min/ml by application of the appropriate extinction co-efficient. Peroxidase values were presented as μmoles NADP formed/min/ml. Both CDNB and peroxidase values could then be converted to final units of specific activity (i.e. μmol product formed /min/mg protein) by including actual protein concentrations of the samples being studied.

All other assays, including Δ^5-androstene-3,17-dione, 4-hydroxynonenal, p-nitrophenyl acetate, p-nitrobenzyl chloride, ethacrynic acid, 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-buten-2-one, were all performed manually at 37°C on a Uvikon 860 spectrophotometer. Further information regarding the conditions under which individual assays using different substrates were performed is presented in Table 2a. The reaction mechanisms for these substrates have been reviewed by both **Habig & Jakoby (1981), and Douglas (1987)**. As for the automated assays, specific activity values were calculated for all of the manual spectrophotometric assays. The change in absorbance (ΔA) was calculated per minute, from which the non-enzymic conjugation rates of substrates with GSH were subtracted. These values were converted to units of μmol/min/ml using the appropriate extinction coefficient (see Table 2a), and subsequently converted to units of specific activity by taking account of protein concentrations of the samples being assayed.

The coefficient of variation obtained using these assays were as follows: 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide, 5%; 4-hydroxynon-2-enal, 6.5%; Δ^5androstene-3,17-

Table 2a: Conditions for Spectrophotometric Assays.

Substrate	[Substrate] (mM)	[GSH] (mM)	pH	Wavelength (nm)	Extinction Coefficient ($mM^{-1}cm^{-1}$)
1-chloro-2,4-dinitrobenzene	1.0	2.0	6.5	340	9.6
cumene hydroperoxide	1.2	1.0	7.6	340	-6.2
Δ^5-androstene-3,17-dione	0.068	0.1	8.5	248	16.3
4-hydroxynon-2-enal	0.1	0.5	6.5	224	13.75
p-nitrophenyl acetate	0.2	0.5	7.0	400	8.8
p-nitrobenzyl chloride	1.0	5.0	6.5	310	1.9
ethacrynic acid	0.2	0.25	6.5	270	5.0
1,2-dichloro-4-nitrobenzene	1.0	5.0	7.5	345	8.5
trans-4-phenyl-3-buten-2-one	0.05	0.25	6.5	290	-24.8

dione, 7.5%; p-nitrophenyl acetate, 9%; p-nitrobenzyl chloride, 10%; ethacrynic acid, 10.5%; 1,2-dichloro-4-nitrobenzene, 15%; *trans*-4-phenyl-3-buten-2-one, 15%.

SDS/Polyacrylamide-gel Electrophoresis (SDS/PAGE)

This was performed essentially as described by **Hayes & Mantle (1986, a, b)** using the discontinuous buffer system developed by **Laemmli (1970)**. Electrophoresis was carried out at room temperature in a Protean II gel electrophoresis system (Bio-Rad) using vertical polyacrylamide slab gels (20 x 20 x 0.1 cm) which comprised a resolving gel of about 16 cm high consisting of 12% (w/v) polyacrylamide containing 0.32% (w/v) NN'-methylenebisacrylamide and 0.375 M Tris/HCl buffer (pH 8.85). The 3.5 cm stacking gel overlaying the resolving gel consisted of 3% (w/v) polyacrylamide containing 0.32% (w/v) NN'-methylenebisacrylamide and 0.125 M Tris/HCl buffer (pH 6.8). This gel was cast following full polymerisation of the resolving gel. SDS (0.1% w/v) was present in both the resolving and stacking gels.

Samples (including cytosolic fractions and purified proteins) were prepared for electrophoresis by the addition of a boiling mix solution which contained 3% (w/v) SDS, 30% glycerol, 2% 2-mercaptoethanol and 0.002% bromophenol blue at a ratio of sample: boiling mix of 2:1, respectively. Samples were heated at 85-90°C for 5-10 min and appropriate aliquots loaded onto the gel. The samples were either stored at -20°C until use, or were subjected to electrophoresis immediately after preparation. Electrophoresis involved running the samples through the stacking gel at a constant 200 volts, followed by the resolving gel at 325 volts. The electrophoresis run was terminated when the bromophenol dye front was approximately 0.5 cm from the bottom edge of the gel. The proteins were visualised by staining the resolving gel for approximately 45 min at room temperature using a 0.2% (w/v) solution of Coomassie brilliant blue R-250 in a water/methanol/acetic acid solution (50:50:7 by volume). The gels were then de-stained in several changes of a solution composed of water/methanol/acetic acid (88:5:7 by volume).

Analytical Isoelectric Focusing (IEF)

Isoelectric focusing (IEF) was performed in a 4.85% (w/v) polyacrylamide slab gel (24 x 11.5 x 0.2 cm) which contained 0.15% (w/v) NN'-methylenebisacrylamide and 5% (v/v) carrier ampholine (pH range 3-10). Electrophoresis was performed using the LKB 2117 Multiphor II system along with the LKB 2303 Multidrive XL 3.5 kV power supply (for details see manufacturer's handbook). The electrode solutions were 1 M orthophosphoric acid at the anode and 1 M sodium hydroxide at the cathode. Samples (5-30 μg protein) were applied to small rectangular pieces (0.5 x 1.0 cm) of filter paper (supplied by the manufacturer) placed at approximately 3 cm from the cathode. Protein pI markers were included in all analyses, comprising amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin-acidic band (pI 6.85), myoglobin-basic band (pI 7.35), lentil lectin-acidic band (pI 8.15), lentil lectin-middle band (pI 8.45), lentil lectin-basic band (pI 8.65) and trypsinogen (pI 9.30).

Focusing was carried out at a constant power of 25 W and was allowed to proceed for 1 h before removal of sample application strips. Focusing was then continued for a further 2.5 h prior to fixing and staining. These proteins were fixed for 0.5 - 1 h in an aqueous solution of sulphosalicylic acid (3.5% w/v) and trichloroacetic acid (11.5% w/v). Following washing in destaining solution consisting of water/ethanol/acetic acid (67:25:8 by volume) for 15-30 min, the gel was stained using a solution of 0.12% (w/v) Coomassie Brilliant Blue R-250 in destaining solution at 60°C for 10 min. Destaining was achieved by using several changes of destaining solution.

Western Blotting

This was carried out using a modified version of the method originally described by **Towbin *et al* (1979)**. Polypeptides were resolved by SDS/PAGE as described above, and immediately following electrophoresis the resolving gels were equilibrated for 20 min in the transfer buffer (25 mM Tris/193 mM glycine) at room temperature to remove SDS. The proteins were electrophoretically transferred from the gel to nitrocellulose paper using a Bio-Rad trans-blot

kit. The transfer was performed for 4 h at a constant current of 0.25 amps using a Bio-Rad 250/2.5 power supply. The nitrocellulose paper was then treated with a 20 mM Tris/HCl-500 mM NaCl (pH 7.5) solution (TBS) which contained 3% skimmed milk overnight to block the remaining protein binding groups.

After washing with TBS to remove the skimmed milk, the nitrocellulose sheets were incubated at room temperature with the first antibody which was diluted in TBS/1% (w/v) gelatin/0.05% (v/v) Tween 20. The antibody titre and time of incubation were both dependent upon the antisera used. Following incubation, the nitrocellulose sheets were washed (2 x 10 min) with TBS/0.05% (v/v) Tween 20 to remove any unbound first antibody. Determination of the polypeptides which had cross-reacted with the first antibody was achieved by using a Bio-Rad goat anti (rabbit IgG) antibody-horseradish peroxide conjugate immunoblot assay kit. The reaction involving the horseradish peroxidase, 4-chloro-1-naphthol and hydrogen peroxide produces a colour-change, the intensity of which gives a qualitative estimation of the amount of any specific protein present.

2.02

(b) Purification of GST Isoenzymes

Preparation of Cytosols

Homogenates [1:4 (w/v)] of bovine tissues were prepared in ice-cold 50 mM Tris/HCl buffer (pH 7.8) that contained 200 mM NaCl (buffer A) using a Wareing blender. Standard centrifugation techniques, involving both low (15000 x g) and high 100 000 x g) speed spins, were used to prepare 100 000 x g supernatant fractions (cytosol) which were subsequently dialysed for 16 h at 4°C against 4 litres of buffer A containing 1 mM 2-mercaptoethanol (buffer B). Following dialysis, the cytosol was re-centrifuged (15000 x g; 30 min; 4°C) to remove precipitated material, and the resulting supernatant subjected to affinity chromatography, with a small portion being retained for enzymic assay or SDS/PAGE analysis.

Exactly the same procedure was followed for the human tissue except for the use of a

hand-held homogeniser rather than a blender during the preparation of the homogenate.

Affinity Chromatography

GST isoenzymes were prepared from each of the cytosols by affinity chromatography on both S-hexylglutathione-Sepharose 6B (S-hex-G-Ag) and glutathione-Sepharose 6B (GSH-Ag), both of which had been equilibrated with buffer B.

S-hexylglutathione-Sepharose 6B (S-hexG-Ag)

For the bovine tissues, dialysed cytosol was applied to columns (1.6 x 30 cm) containing S-hex-Ag, and non-specifically adsorbed protein removed by extensive washing (approximately 10 column volumes) with buffer B. Specifically-bound material was eluted using a gradient elution of the affinity matrix using a 0-0.25 mM gradient of S-hexylglutathione in buffer A, as described by **Hayes (1988)**. Fractions (6.5 ml) were collected throughout the elution, which were subsequently used in further analytical procedures.

For the human adrenocortical tissue, dialysed cytosol was applied to much smaller S-hexG-Ag columns (1 x 4 cm), and specifically-bound proteins eluted in a single step using 5 mM S-hexylglutathione in buffer A, collecting 1 ml fractions during the elution.

Glutathione-Sepharose 6B (GSH-Ag)

This affinity matrix was employed to purify GST isoenzymes remaining in the cytosol following affinity chromatography on S-hexG-Ag. Therefore, following a low-speed spin (15000 x g; 30 min; 4°C), the flow-through from the S-hexG-Ag column was applied to GSH-Ag columns (1.6 x 30 cm). Non-specifically adsorbed protein was removed by extensive washing with buffer B before specifically-bound material was eluted using a solution of 40 mM GSH in 200 mM Tris/HCl buffer (pH 9.0). Fractions (6.5 ml) were collected throughout for further analytical procedures.

For the human adrenal cortex cytosol, flow-through from the S-hexG-Ag column was also re-applied to a column containing GSH-Ag (1 x 4 cm), and specifically-bound protein eluted as described above, collecting 1 ml fractions during the elution.

Other Chromatographic Methods

Ion-Exchange Chromatography

Several affinity-purified GST isoenzymes were resolved further on a Pharmacia F.P.L.C. gradient programmer GP-250 using the Pharmacia mono-disperse anion-exchange column, mono-Q. All chromatographic runs used linear gradients and employed 0.5 M sodium chloride in the limit buffer. Tris/HCl buffers were used for all samples at various pH's (see Results) which also contained 1 mM 2-mercaptoethanol. All samples were dialysed against the running buffer and filtered using a Millipore 0.45 μm filter prior to injection onto the column. A flow-rate of 0.75 mL/min was used throughout, and the eluate monitored at 280 nm. Fractions (0.75 ml) were collected during the elution and subsequently assayed for enzyme activity and analysed by SDS/PAGE.

Reverse-Phase h.p.l.c.:

The system employed was from Waters Associates (Instruments), Northwich Cheshire, U.K., and comprised two model 510 pumps, a model 680 automated controller, a model 481 Lambda Max absorbance detector and a model U6K universal injector. The Waters μ- Bondapak C_{18} column (10 μm particle size; column size 0.39 x 30.0 cm) was developed at 1 ml/min using linear gradients of acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid, with pump A delivering 40% acetonitrile and pump B 70%. The eluate was monitored continuously at 220 nm and peaks were collected manually during each run. All samples for h.p.l.c. were centrifuged at 10000 x g for 5 min to remove precipitated material before injection onto the column. For all applications, the gradient controller was programmed to include a 5 min "loading time", during which the sample was applied isocratically (i.e. 100% solvent A) and the flow-rate increased linearly from 0.1 to 1.0 ml/min. Further details of the actual gradients employed for different samples will be given in the Results section.

2.02

(c) Experiments Involving Primary Cultures of Bovine Adrenocortical Cells

Preparation of Primary Cultures

The preparation of primary cultures of bovine adrenocortical cells has been extensively described elsewhere **(Walker *et al.*, 1988; Lightly, 1991)**. In essence, this technique enables the preparation of ZF/ZR cells which are free of contamination by zona glomerulosa cells, and therefore provides an excellent *in vitro* system for investigating adrenocortical cell metabolism. In this method, careful sectioning of the whole adrenal gland using a Staddie-Riggs microtome enabled the collection of 100 μm slices consisting mainly of ZF/ZR tissue into Earle's balanced salt solution (EBS) containing 0.2% BSA. These tissue slices were then digested using EBS/2% BSA which contained 1.5-2.0 mg/ml collagenase type 1. Following several centrifugation steps and filtration through different nylon gauzes to remove undigested tissue and excess debris, further purification of ZF/ZR cells was achieved by using the column-filtration method of **McDougall *et al* (1979)**. Using this step, not only were zona glomerulosa and medullary cells removed from the cell suspension but also contaminating red blood cells and subcellular debris. These columns contained approximately 5 ml Sephadex G10 (40-120 μm bead size) which had approximately 2 ml Sephadex G50 layered on top. The Sephadex G10 was used because of its bead size which was found to be ideal for specifically trapping ZF/ZR cells in this preparation, whereas the other cells passed straight through the column.

On harvesting the cells from the gel by filtration of the gel suspension through a 30 μm gauze, a cell suspension was obtained which consisted almost entirely of ZF/ZR cells. The cells were then plated out at the appropriate density in Ham's F10 growth medium containing various additives such as CPSR-5, penicillin, streptomycin and amphoterin B. For the work described in this thesis, 75 cm^3 Costar tissue culture flasks were used in which the cells were plated out at 10 million per flask in 15 ml of growth medium. Cells were maintained in a Scotlab VSL incubator at 37°C in an atmosphere of 5% CO_2 and 100% humidity. Phase-contrast light microscopy showed that the cells were attached to the culture flask surface within 12 h of plating out: the

cells initially were round-shaped, although at later times in culture they adopted a more flattened and elongated morphology.

Agonist Studies on Cultured Cells

The agonists used in this project were $ACTH_{1-24}$ and angiotensin II (AII). These were made up to the appropriate concentration in Ham's F10 containing 0.2% BSA. Routinely, the overlying growth medium was removed from each flask and washed (x 2) with 10 ml F10/0.2%BSA before initiation of the experiment by adding the agonist in the same solution. All agonist experiments were carried out on day 3 of culture for reasons which will be discussed in the Results section. The flasks were then incubated at 37°C in an atmosphere of 5% CO_2. Following stimulation of the cells for the appropriate time (see Results), the overlying medium was removed and the flasks washed (x 3)with 15 ml of a solution of ice-cold phosphate buffered saline (PBS), ready for the preparation of cell cytosols.

Preparation of Cytosol from Bovine Adrenocortical Cells in Primary Culture

The cells were detached from each flask surface by the addition of a 0.01% trypsin-EDTA solution (10 ml) and subsequent incubation at 37°C for 10 min. Cells were then harvested from each flask using excess PBS (15-20 ml), and centrifuged at 450 x g for 30 min. The cell pellet obtained was re-suspended in excess (20 ml) Ham's F10 including 10% CPSR-5: this has the dual effect of both diluting and inhibiting the trypsin since the CPSR-5 contains a trypsin inhibitor.

Following another centrifugation step (450 x g; 30 min), the cell pellet was re-suspended in excess PBS (20 ml), which had the effect of diluting out the proteins present in the CPSR-5, necessary because one of the analytical methods to be used later on the cytosol was SDS/PAGE. The PBS cell suspension was centrifuged again (450 x g; 30 min), after which the cell pellet was re-suspended in a small volume (1 ml) of PBS. The resulting cell suspension was disrupted by sonication (on ice) using an MDH Soniprep ultrasonic frequency emitter (2 x 2 sec frequency emissions), and the cell homogenate subjected to ultracentrifugation (100 000 x g; 1 h; 4°C). The supernatant from this step (cytosol) was carefully removed from the tube and used for enzymic

assays involving different GST substrates along with SDS/PAGE analysis.

Chapter 3: RESULTS.

3.01 Preliminary Examination of Bovine GSTs

The range of GST isoenzymes expressed in different bovine organs was studied initially using S-hexylglutathione-Sepharose 6B (S-hexG-Ag); the alpha, pi and mu-class GST can be eluted sequentially from this affinity matrix using gradients of S-hexylglutathione. S-hexG-Ag was selected as opposed to glutathione-Sepharose 6B (GSH-Ag) because of its ability to resolve bovine GSTs **(Hayes *et al.*, 1989b)**. The adrenal cortex was the organ most extensively investigated because the study of adrenocortical GST expression was to form the basis of the thesis. Nevertheless, for comparison purposes, cytosols from several other bovine organs were also subjected to affinity chromatography on S-hexG-Ag.

(i) Bovine Adrenal Cortex

The GST isoenzymes in cytosol obtained from bovine adrenal cortex tissue were bound to S-hexG-Ag and eluted using a gradient of 0-0.25 mM S-hexylglutathione (Fig. 3.01.A(i)). Enzyme analysis of individual fractions collected during the gradient elution revealed 3 peaks of GST activity: an initial peak consisting of fractions 11-18, a second peak consisting of fractions 21-28, and a third peak, or shoulder, which was observed immediately following peak 2 from fraction 30 onwards. SDS/PAGE analysis of selected fractions across this profile revealed the GST subunit composition of these 3 peaks of activity. The first peak was found to consist of 2 subunits (fractions 13 and 17, lanes 1 and 2 of Fig. 3.01.A(i)): a slower-migrating subunit with an approximate Mr of 27 000, and a faster-migrating subunit with an approximate Mr of 25 900. The second peak was found to comprise one main subunit of Mr 24 900 (fractions 23 and 27, lanes 3 and 4 of Fig. 3.01.A(i)), and as this peak merged into the shoulder part of the profile, this faster-migrating subunit gradually disappeared, with the appearance of 2 distinct polypeptides of Mr 27 300 and Mr 26 100 (fractions 29, 32, 34; lanes 5,6,7 of Fig. 3.01.A(i)). Elution with 5 mM S-hexylglutathione, following gradient elution, resulted in the purification of a faster-migrating polypeptide (Fig. 3.01.A(i), lane 8) with an apparent Mr of 23 800.

In order to determine the GST subclass to which each bovine enzyme belonged, the technique of immunoblotting was employed, using antibodies which had been raised against different GST subunits in the rat (Fig. 3.01.A(ii)). Each fraction from the S-hexG-Ag profile

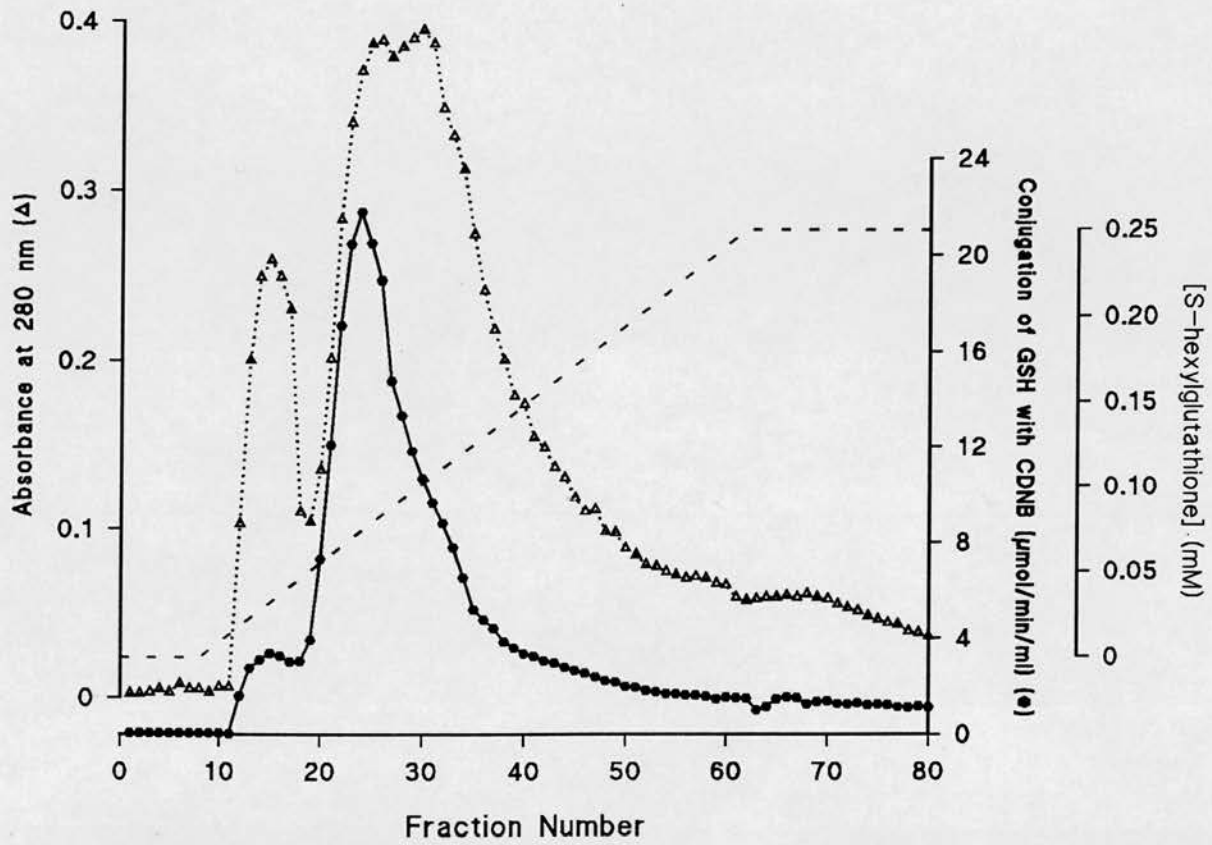
Figure 3.01.A (i):

Gradient Elution of Bovine Adrenal Cortex GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine adrenal cortex tissue were applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The bound GST isoenzymes were eluted using a gradient of S-hexylglutathione (0-0.25 mM), collecting 6.5 ml fractions throughout. The remaining material bound to the column was eluted in a single step using a solution of 5 mM S-hexylglutathione. Each fraction was assayed for GST activity (using CDNB as the substrate) and protein concentration, and these values used to plot the elution profile shown opposite.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel obtained (shown opposite) was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 13; lane 2, fraction 17; lane 3, fraction 23; lane 4, fraction 27; lane 5, fraction 29; lane 6, fraction 32; lane 7, fraction 34; lane 8, protein eluted using 5 mM S-hexylglutathione.

Figure 3.01(A)


Absorbance at 280 nm (Δ)
0.4
0.3
0.2
0.1
0
Conjugation of GSH with CDNB [μmol/min/ml] (•)
24
20
16
12
8
4
0
[S-hexylglutathione] (mM)
0.25
0.20
0.15
0.10
0.05
0
0 10 20 30 40 50 60 70 80
Fraction Number

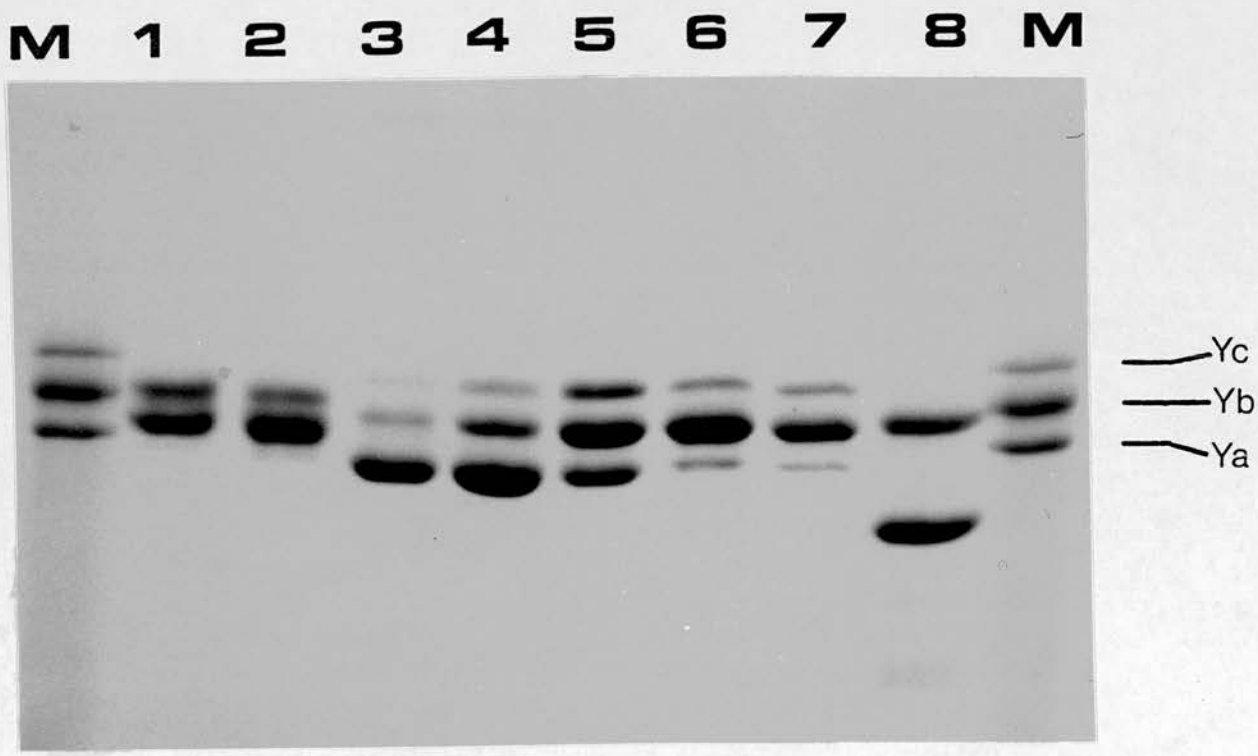

M 1 2 3 4 5 6 7 8 M
Yc
Yb
Ya

Figure 3.01.A (ii)

Immunoblotting of Affinity-Purified GSTs from Bovine Adrenal Cortex Using Anti(-Rat GST) Antisera.

The same fractions described in fig. 3.01.A (i) for affinity-purified adrenal cortex GSTs were used in immunoblots involving various anti(-Rat GST) antisera. Blots A, B, C and D thus correspond to anti(-Rat Ya), anti(-Rat Yf), anti(-rat Yb_1) and anti(-Rat Yb_2) antisera respectively; lanes 1-7 in each blot correspond to fractions 13, 17, 23, 27, 29, 32 and 34 from the S-hexG-Ag gradient elution shown in Fig. 3.01.A (i); lane 8 corresponds to the protein eluted with 5 mM ligand, and lane "M" contains the rat liver GST marker.

Figure 3.01.Aii

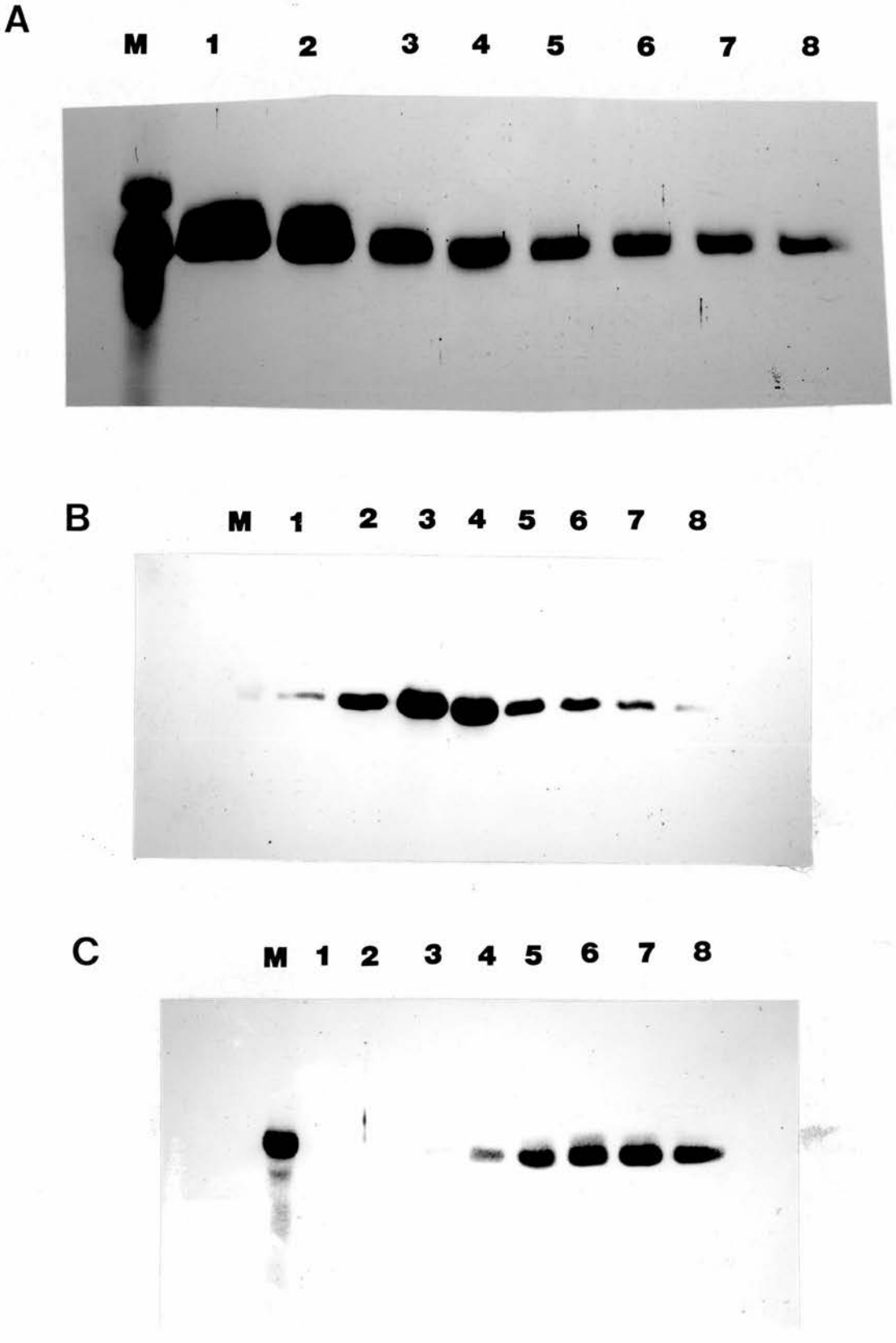


Figure 3.01.Aii

D

M 1 2 3 4 5 6 7 8

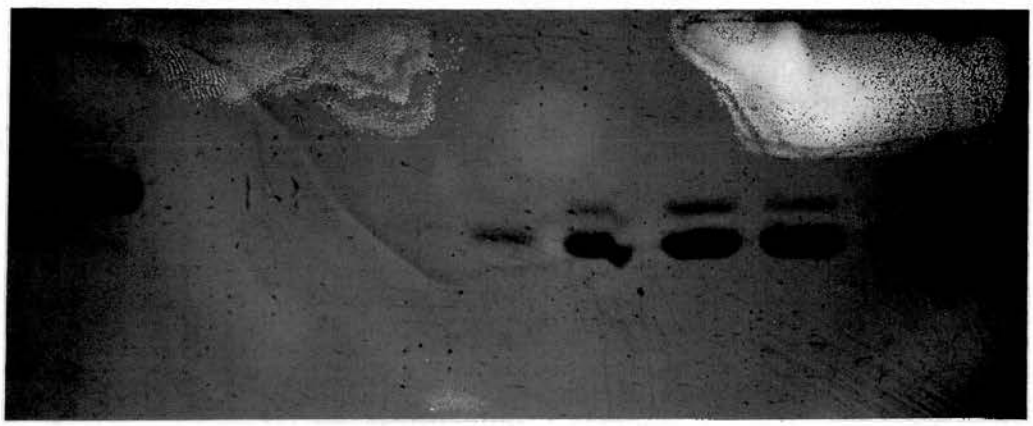
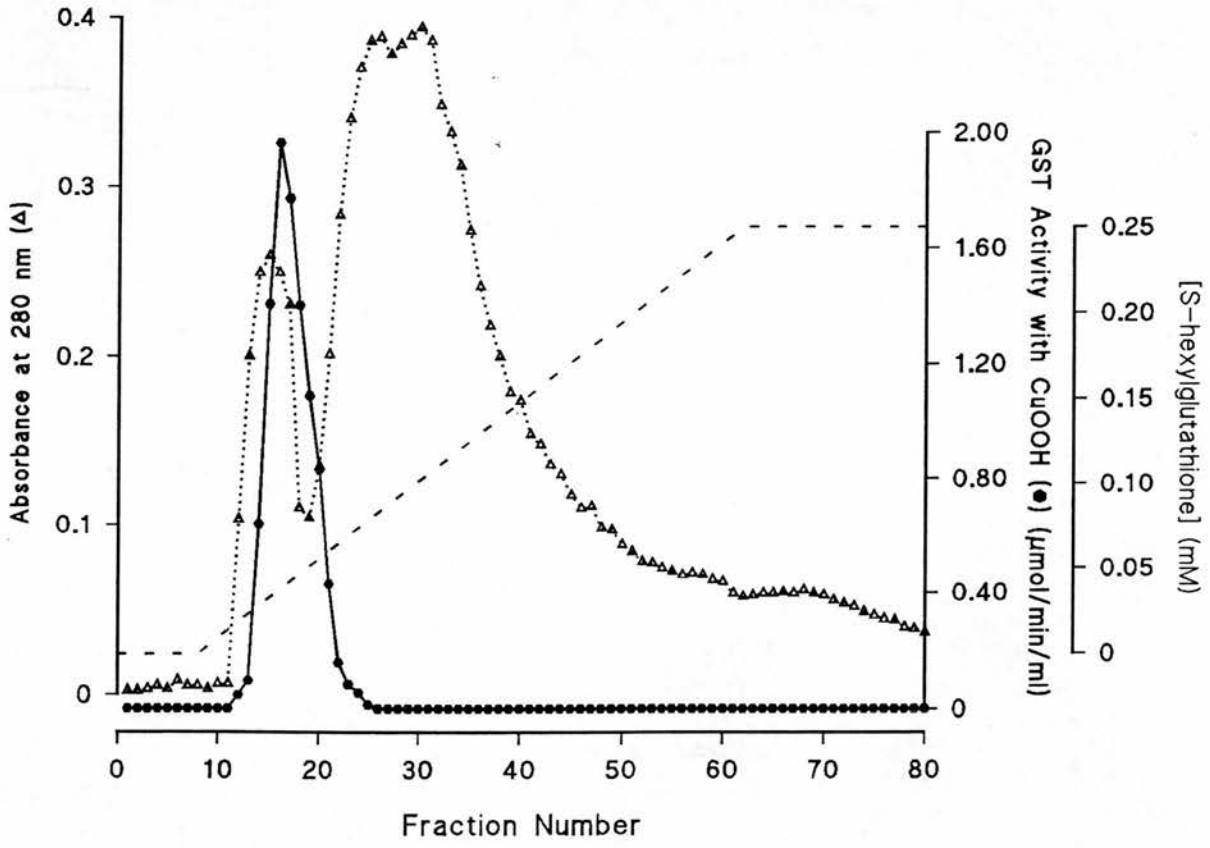


Figure 3.01(B)



Gradient Elution of Bovine Adrenal Cortex GST on S-Hexylglutathione-Sepharose 6B.

Fractions collected during gradient elution of bovine adrenal cortex GST from S-hexylglutathione-Sepharose 6B were each assayed for activity with cumene hydroperoxide (see "methods"). The values obtained for each fraction were plotted alongside those for protein concentration.

analysed by SDS/PAGE in Fig. 3.01.A(i) was therefore used in Western blots with antibodies which had been raised separately against rat GST Ya, Yc, Yb_1, Yb_2 and Yf subunits. Results from these blots (A-D, Fig. 3.01.A(ii)) showed lanes 1 and 2 to have marked cross-reactivity with the anti-(rat Ya) antiserum (blot A), although only very faint non-specific cross-reactivity using the anti-(rat Yc) antiserum (results not included). Furthermore, both bands apparent in lanes 1 and 2 of the SDS/PAGE gel appeared to cross-react with the anti-(rat Ya) antiserum, suggesting both of these GST subunits to be Ya-like. Immunoblotting using the anti-(rat Yf) antisera revealed marked cross-reactivity with a single band in lanes 3 and 4 (blot B), corresponding to those fractions making up the second peak of activity in the elution profile. Lanes 5-8 showed marked cross-reactivity with a single band using the anti-(rat Yb_1) antisera (blot C), although the anti-(rat Yb_2) antisera showed only faint cross-reactivity with both bands present in these fractions (lanes 5-8, Fig. 3.01.A(ii)). The results from immunoblotting were thus consistent with there being GST isoenzymes from each of the 3 classes (alpha, mu and pi) in bovine adrenal cortex. These 3 classes could be purified into 3 main peaks on S-hexG-Ag: an initial peak containing mainly alpha-class GST, a second peak consisting of fractions containing mainly pi-class GST, and finally a shoulder of activity on the second peak, consisting of fractions containing mainly mu-class GST.

The results of immunoblotting were further supported by assaying each of the S-hexG-Ag purified fractions for GST activity using a range of model GST substrates which are known to be preferentially metabolised by the different classes of GST (see "Introduction"). The "general" substrate, CDNB, showed highest activity with those fractions containing mainly pi-class GST, with fractions containing both alpha- and mu-class pools showing approximately half as much activity with this substrate (Table 3.01.A). The fractions containing alpha-class GST (see Fractions 13 and 17; lanes 1 and 2 in Fig. 3.01.A), however, showed highest activity using cumene hydroperoxide as the substrate. This was also the case when Δ^5 androstene-3,17-dione and 4-hydroxynon-2-enal were used as substrates. The activities of the affinity-purified GST fractions towards p-nitrobenzyl chloride and p-nitrophenyl acetate were less well-defined: p-nitrobenzyl chloride showed little activity with the alpha-class enzyme(s), although both pi- and mu-class GST

Table 3.01(A)

Specific Activities of Various GST Isoenzyme Pools Purified from Bovine Adrenal Cortex on S-Hexylglutathione-Sepharose 6B Using Different GST Substrates.

S-hexyl Pool Number	Specific Activity (all μmol/min/mg protein)						
	CDNB	CuOOH	ADD	NON	NBC	NPA	EA
(1)	7.12	8.18	0.40	2.43	0.25	0.36	N.S.
(2)	8.21	5.30	0.34	1.90	0.14	0.34	N.S.
(3)	13.47	0.48	N.S.	0.72	N.S.	N.S.	N.S.
(4)	15.26	0.17	0.04	0.77	0.53	0.02	0.13
(5)	14.08	0.27	0.04	0.47	1.04	0.13	0.18
(6)	5.86	0.50	N.S.	0.31	1.19	0.18	0.24
(7)	6.14	0.45	N.S.	0.39	0.96	0.24	0.10
(8)	6.59	0.41	N.S.	0.53	0.85	0.37	N.S.

Abbreviations:

CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5-androstene-3,17-dione; NON, 4-hydroxynon-2-enal; NBC, p-nitrobenzyl chloride; NPA, p-nitrophenyl acetate; EA, ethacrynic acid; N.S., not significant.

The S-hexyl pool numbers correspond to those fractions described during SDS/PAGE analysis of certain fractions purified on S-hexylglutathione-Sepharose 6B (see Fig. 3.01.A).

enzymes appeared to show significant activity with this substrate. Both alpha- and mu-class GSTs appeared to be active with p-nitrophenyl acetate, although the pi-class GST did not show any activity towards this substrate. The GST substrate, ethacrynic acid, did not show any activity with the alpha-class GST enzyme, although the pi-class GST did (as expected), with a small amount of activity towards the mu-class GST. No significant activity could be detected for any of the affinity-purified GST fractions with the mu-class specific substrates, 1,2-dichloro-4-nitrobenzene and *trans*-4-phenyl-3-buten-2-one, and the results are not, therefore, shown in table 3.01.A.

As further confirmation for the presence of alpha-class GST in each individual fraction eluted from the S-hexG-Ag column, cumene hydroperoxide was used in an automated assay (see "Methods"). By plotting the values obtained alongside those for protein concentration, the corresponding elution profile gave an indication of those fractions containing alpha-class GST (Fig. 3.01.B). One peak was observed at almost the same position as the first peak in the elution profile using CDNB as the substrate, further confirming that the GSTs in those fractions making up peak 1 in the elution of GSTs from bovine adrenal cortex cytosol belong to the alpha-class. This assay, using cumene hydroperoxide as the substrate, was subsequently used as an alternative to CDNB in detecting alpha-class GST in all future studies of GSTs in other bovine organs.

(ii) GST Isoenzymes in Other Bovine Organs

A number of other bovine organs were used to purify cytosolic GSTs by affinity chromatography on S-hexG-Ag. The elution profile for bovine liver GSTs appeared slightly more complex than that for the adrenal cortex, consisting of a least 3 main peaks of activity (Fig. 3.01.C). SDS/PAGE analysis of selected fractions across this profile revealed a correspondingly greater number of distinct polypeptides (Fig. 3.01.C). The first peak (Fractions 13-25) consisted of polypeptides which had the same electrophoretic mobility as those alpha-class enzymes found in the first peak eluted using cytosol from the adrenal cortex, although for the liver there appeared to be at least 4 distinct bands in this region (see lane 3, Fig. 3.01.C). The third peak appeared to consist of the same two bands of mu-class GST enzymes, as reported in the adrenal cortex, although there was a hint (see lane 9, Fig. 3.01.C) that the faster of the two mu-class bands might

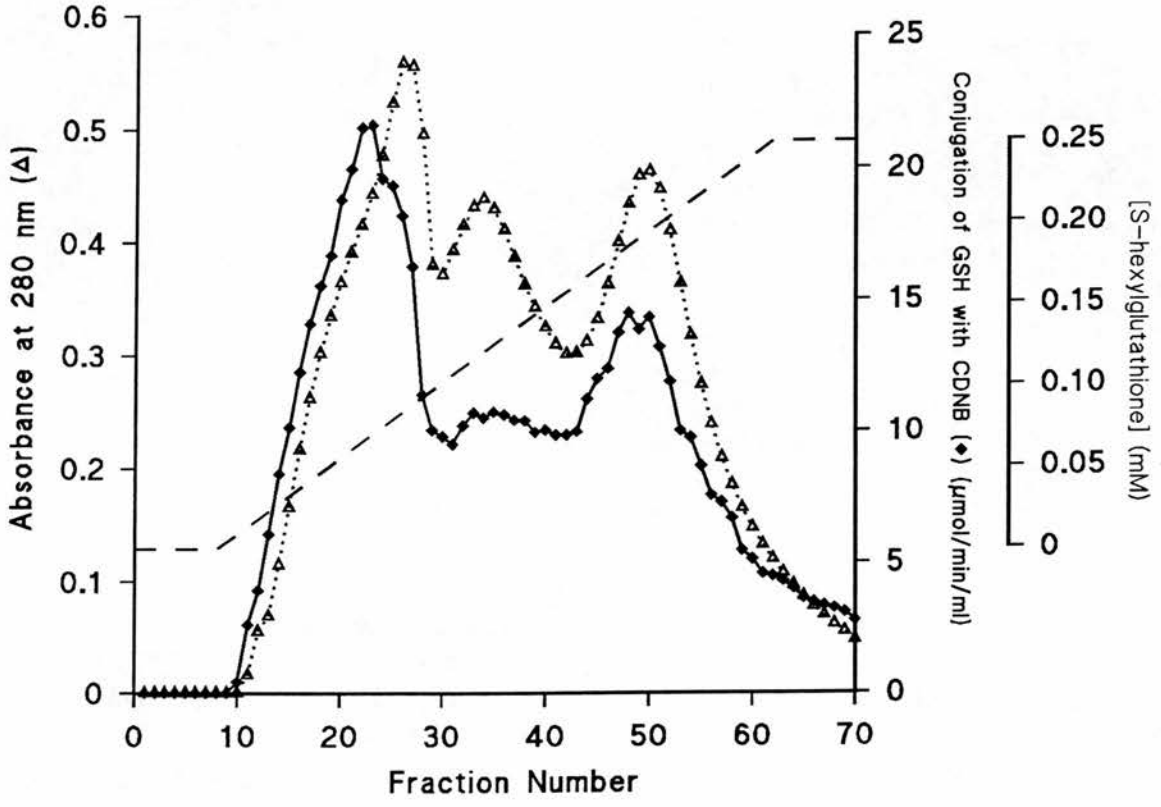
Figure 3.01(C)

Gradient Elution of Bovine Liver GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine liver were prepared (see "methods") and applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The bound GST isoenzymes were eluted using a gradient of S-hexylglutathione (0-0.25 mM) and collected in 6.5 ml fractions. Each fraction was assayed for GST activity (using CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 14; lane 2, fraction 20; lane 3, fraction 24; lane 4, fraction 28; lane 5, fraction 33; lane 6, fraction 36; lane 7, fraction 40; lane 8, fraction 46; lane 9, fraction 51.

Figure 3.01(C)


Absorbance at 280 nm (Δ)
Conjugation of GSH with CDNB (♦) (μmol/min/ml)
[S-hexylglutathione] (mM)
Fraction Number
0.6
0.5
0.4
0.3
0.2
0.1
0
25
20
15
10
5
0
0.25
0.20
0.15
0.10
0.05
0
0 10 20 30 40 50 60 70

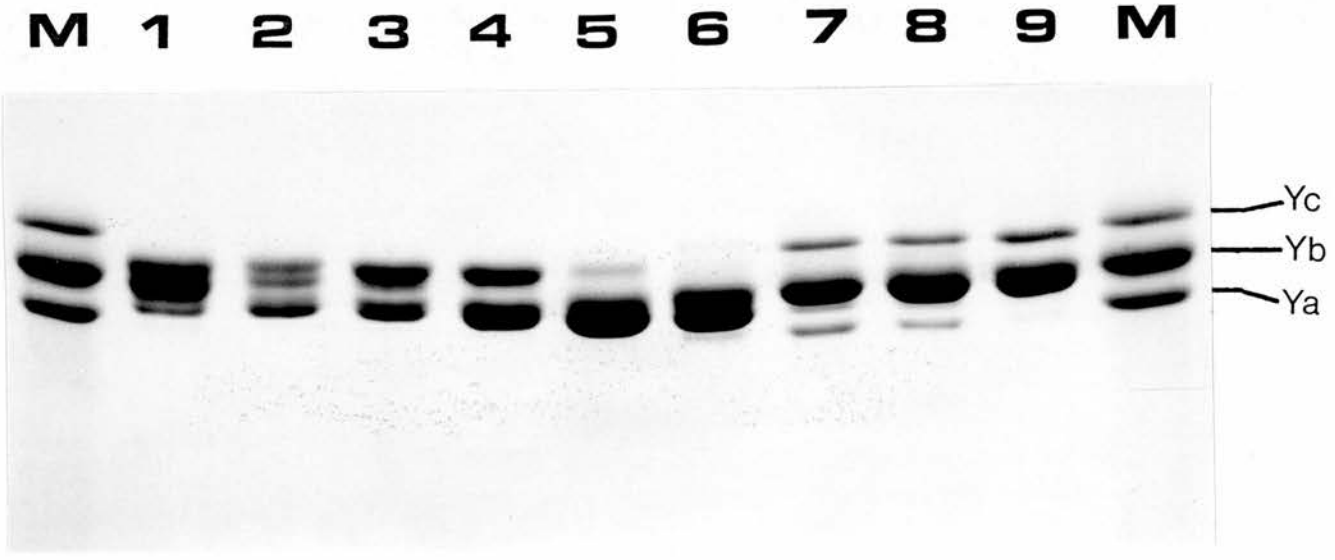
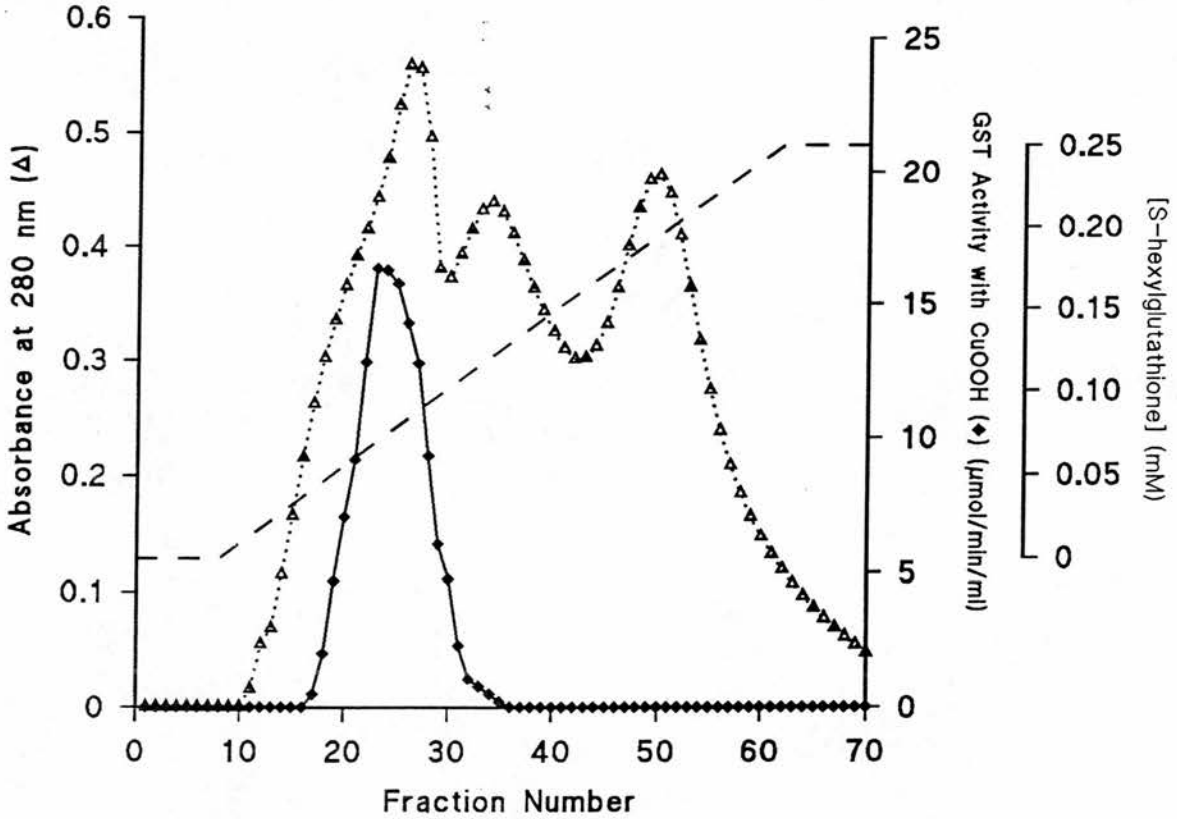

M 1 2 3 4 5 6 7 8 9 M
Yc
Yb
Ya

Figure 3.01(D)



Gradient Elution of Bovine Liver GST on S-Hexylglutathione-Sepharose 6B.

Fractions collected during gradient elution of bovine liver GST from S-hexylglutathione-Sepharose 6B were each assayed for activity with cumene hydroperoxide (see "methods"). The values obtained for each fraction were plotted alongside those for protein concentration.

consist of two polypeptides. The second peak of activity in the liver GST elution profile, which was significantly smaller than the other two, appeared to be the intermediate region between alpha-class and mu-class peaks, containing small amounts of the pi-class subunit seen at much higher levels in the adrenal cortex. Automated analysis of each fraction for GST activity with cumene hydroperoxide revealed the first peak only to possess activity with this substrate (Fig. 3.01.D). This is consistent with the observation made from the SDS/PAGE gel that peak 1 in the elution profile (Fig. 3.01.C) comprised those fractions containing alpha-class GST.

Bovine testes were also studied. The elution profile again revealed three distinct peaks of activity using CDNB as the substrate. Overall, the profile was similar to the liver, although peak 2 showed relatively higher activity (Fig. 3.01.E) in the testes. SDS/PAGE analysis of selected fractions across this profile displayed similarities to the corresponding SDS/PAGE gel from the adrenal cortex. However, the first peak from the testes appeared to contain a polypeptide very early on in the gradient which had an electrophoretic mobility Mr of 26 500 (lane 1, Fig. 3.01.E). The second peak of activity consisted of the fast-migrating pi-class GST with an electrophoretic mobility very similar to that found in the adrenal cortex (lane 5, Fig. 3.01.E). The third peak consisted of GST subunits which also showed very similar electrophoretic mobilities to those found in the adrenal cortex (lanes 6, 7 and 8; fig. 3.01.E). The presence of alpha-class GST in peak 1 is further corroborated by the results of the glutathione peroxidase assay on each fraction across the elution profile (Fig. 3.01.F). Interestingly, for bovine testes GST the corresponding activity peak not only coincided with peak 1 of the profile with CDNB as the substrate, but also consisted of two distinct regions: fractions 11-20 retained the highest activity, although fractions 21-27 also showed significant activity with cumene hydroperoxide as the substrate.

Bovine lung was also found to contain relatively high levels of alpha-class GST (Fig. 3.01.G). The elution profile using CDNB as the substrate revealed two main peaks of activity, with a shoulder of activity after the second peak. SDS/PAGE analysis showed peak 1 (Fractions 11-17) to consist of one polypeptide with the same apparent electrophoretic mobility as the slower-migrating alpha-class GST in the adrenal cortex (approximate Mr of 27 000). The elution profile using cumene hydroperoxide as the substrate again confirmed that those fractions making up

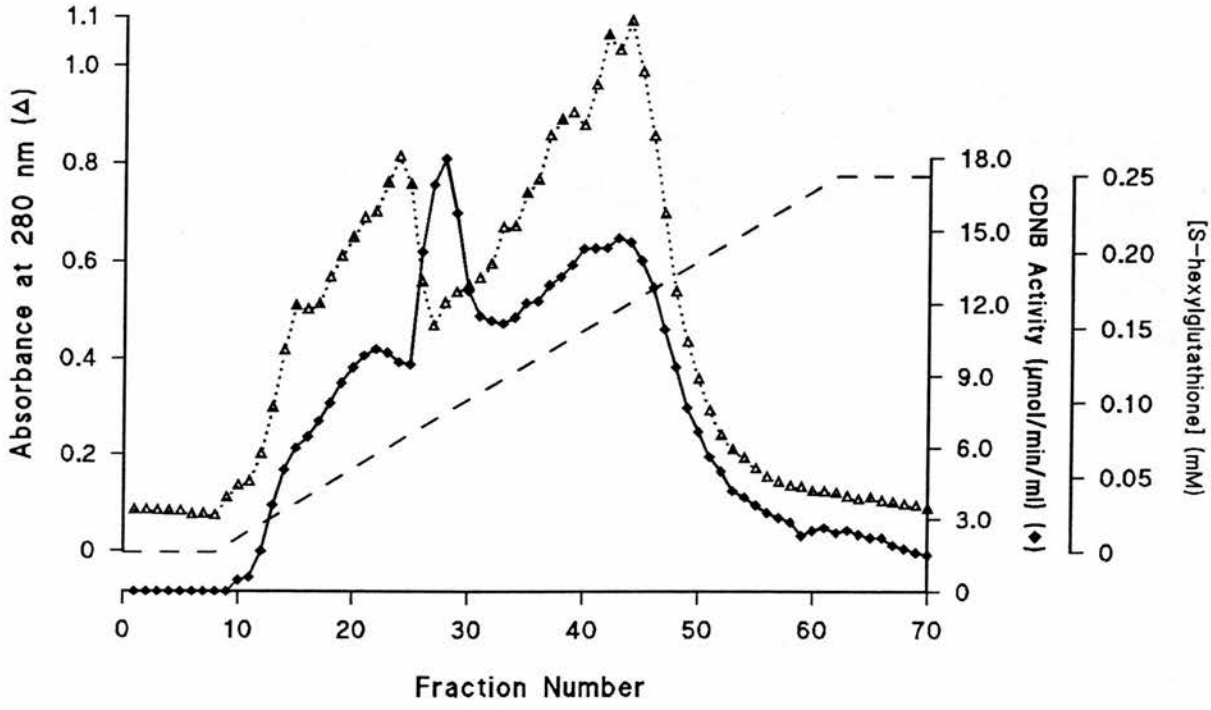
Figure 3.01(E)

Gradient Elution of Bovine Testes GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine testes were prepared (see "methods") and applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The GSTs subsequently bound to this affinity matrix were eluted using a gradient of S-hexylglutathione (0-0.25 mM) and collected in 6.5 ml fractions. Each fraction was assayed for GST activity (with CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels .The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 13; lane 2, fraction 16; lane 3, fraction 19; lane 4, fraction 22; lane 5, fraction 25; lane 6, fraction 33; lane 7, fraction 43; lane 8, fraction 52.

Figure 3.01(E)


Absorbance at 280 nm (Δ)
1.1
1.0
0.8
0.6
0.4
0.2
0
18.0
15.0
12.0
9.0
6.0
3.0
0
CDNB Activity (μmol/min/ml) (♦)
0.25
0.20
0.15
0.10
0.05
0
[S-hexylglutathione] (mM)
0
10
20
30
40
50
60
70
Fraction Number

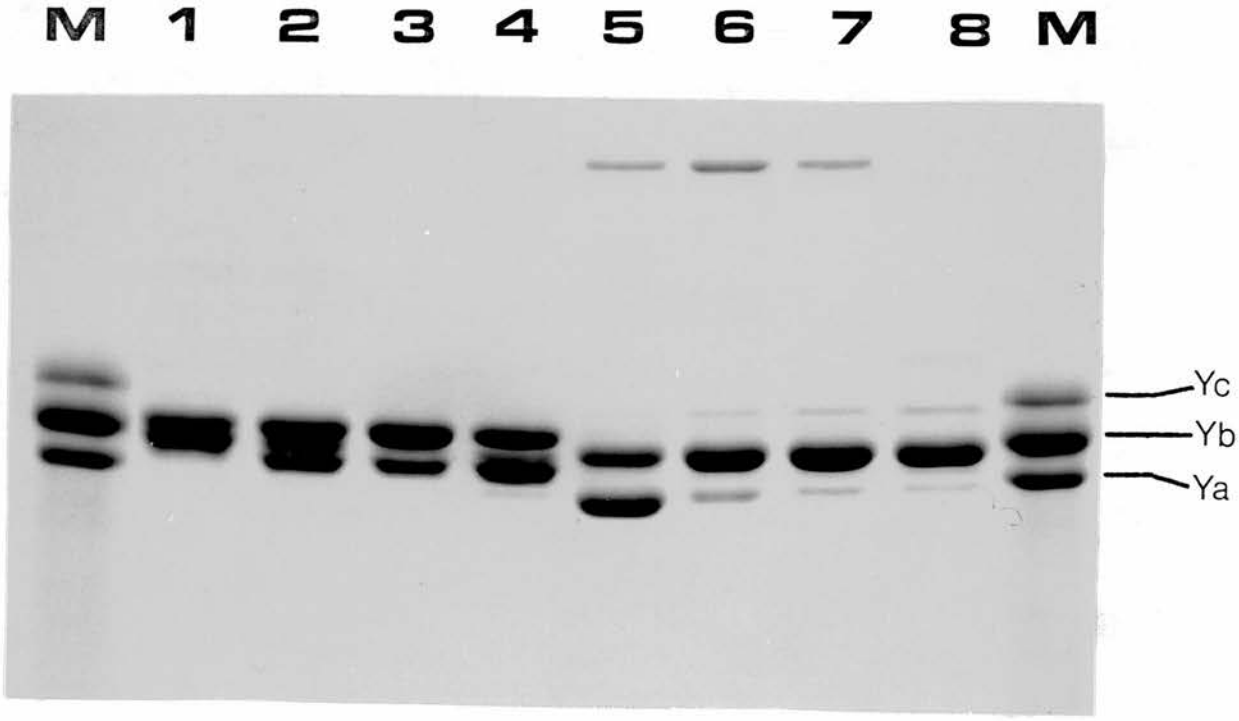
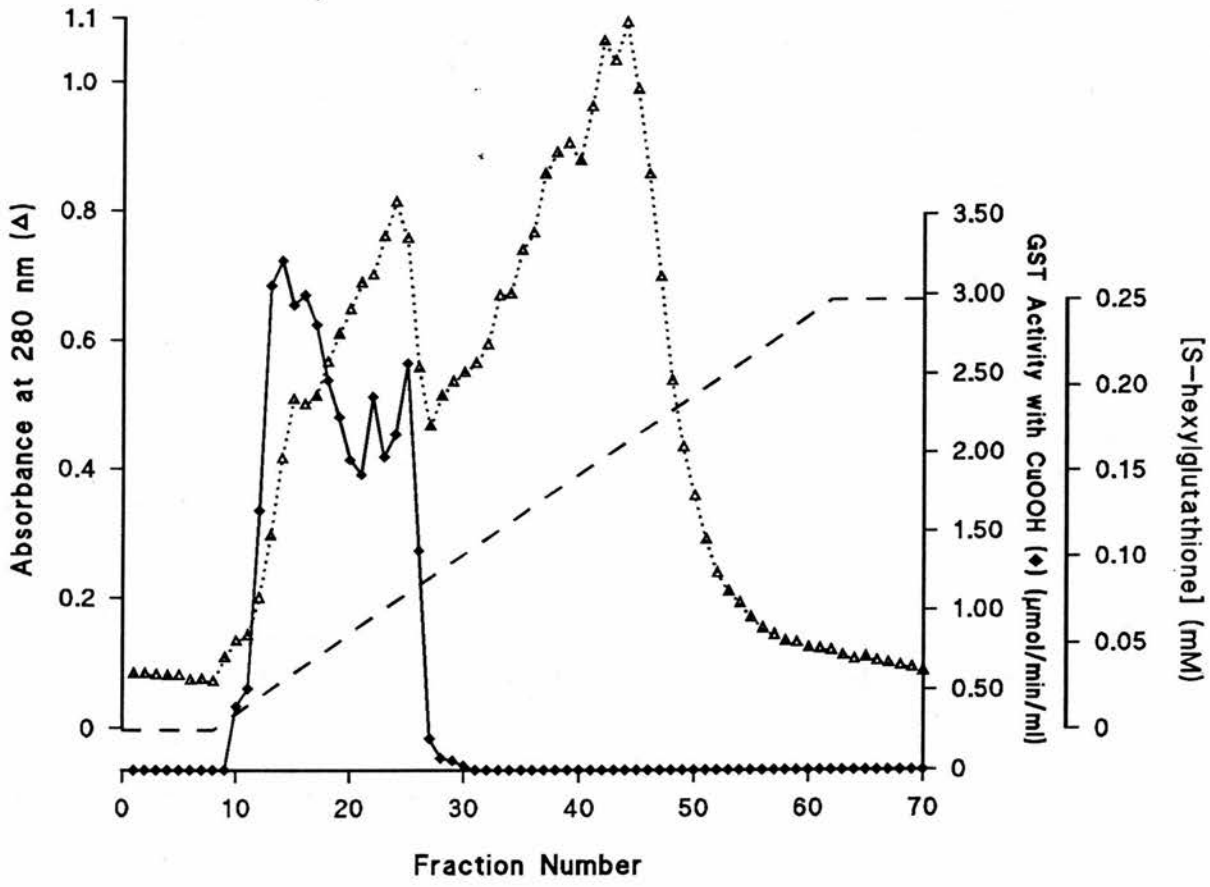

M
1
2
3
4
5
6
7
8
M
Yc
Yb
Ya

Figure 3.01(F)



Gradient Elution of Bovine Testes GST on S-Hexylglutathione-Sepharose 6B.

Fractions collected during gradient elution of bovine testes GST from S-hexylglutathione-Sepharose 6B were each assayed for activity with cumene hydroperoxide (see "methods"). The values obtained for each fraction were plotted alongside those for protein concentration.

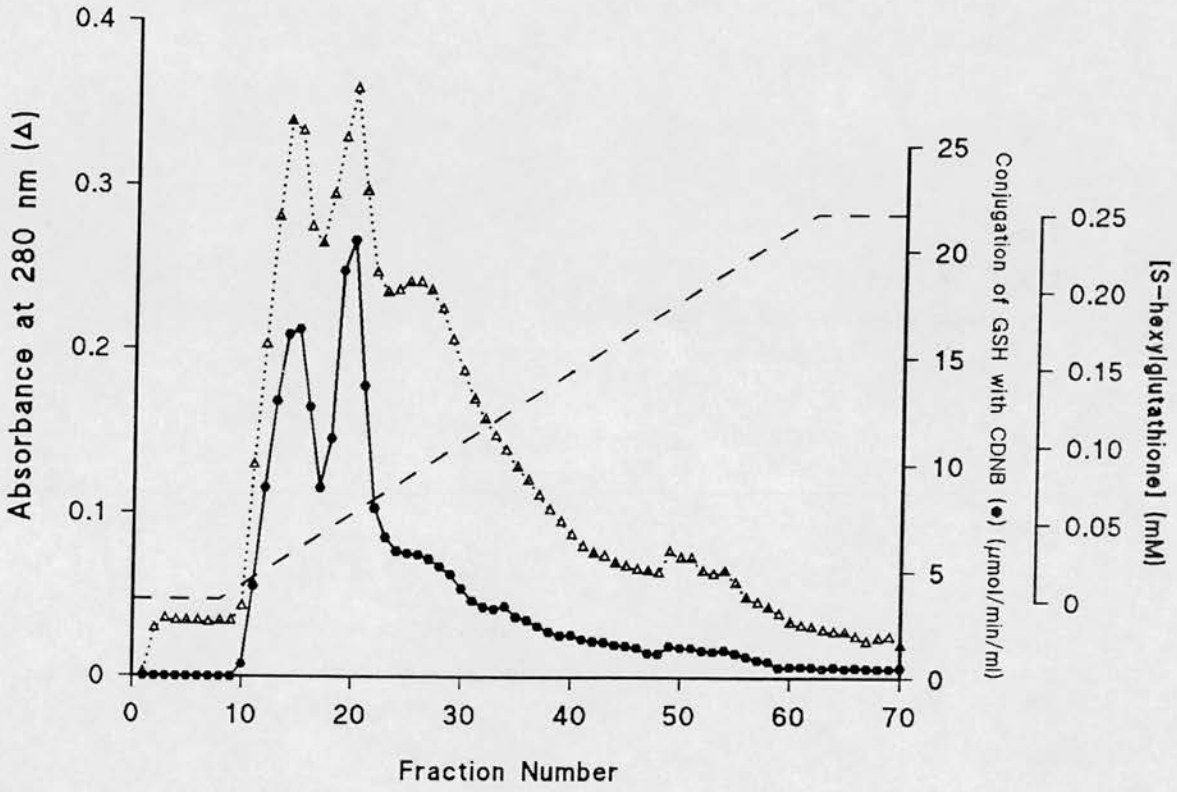
Figure 3.01(G)

Gradient Elution of Bovine Lung GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine lung tissue were prepared (see "methods") and applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The GST isoenzymes bound to this affinity matrix were eluted using a gradient of S-hexylglutathione (0-0.25 mM), and 6.5 ml fractions collected. Each fraction was assayed for GST activity (with CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 13; lane 2, fraction 15; lane 3, fraction 17; lane 4, fraction 20; lane 5, fraction 22; lane 6, fraction 25; lane 7, fraction 27.

Figure 3.01(G)


Absorbance at 280 nm (Δ)
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Fraction Number
25
20
15
10
5
0
Conjugation of GSH with CDNB (•) (μmol/min/ml)
0.25
0.20
0.15
0.10
0.05
0
[S-hexylglutathione] (mM)

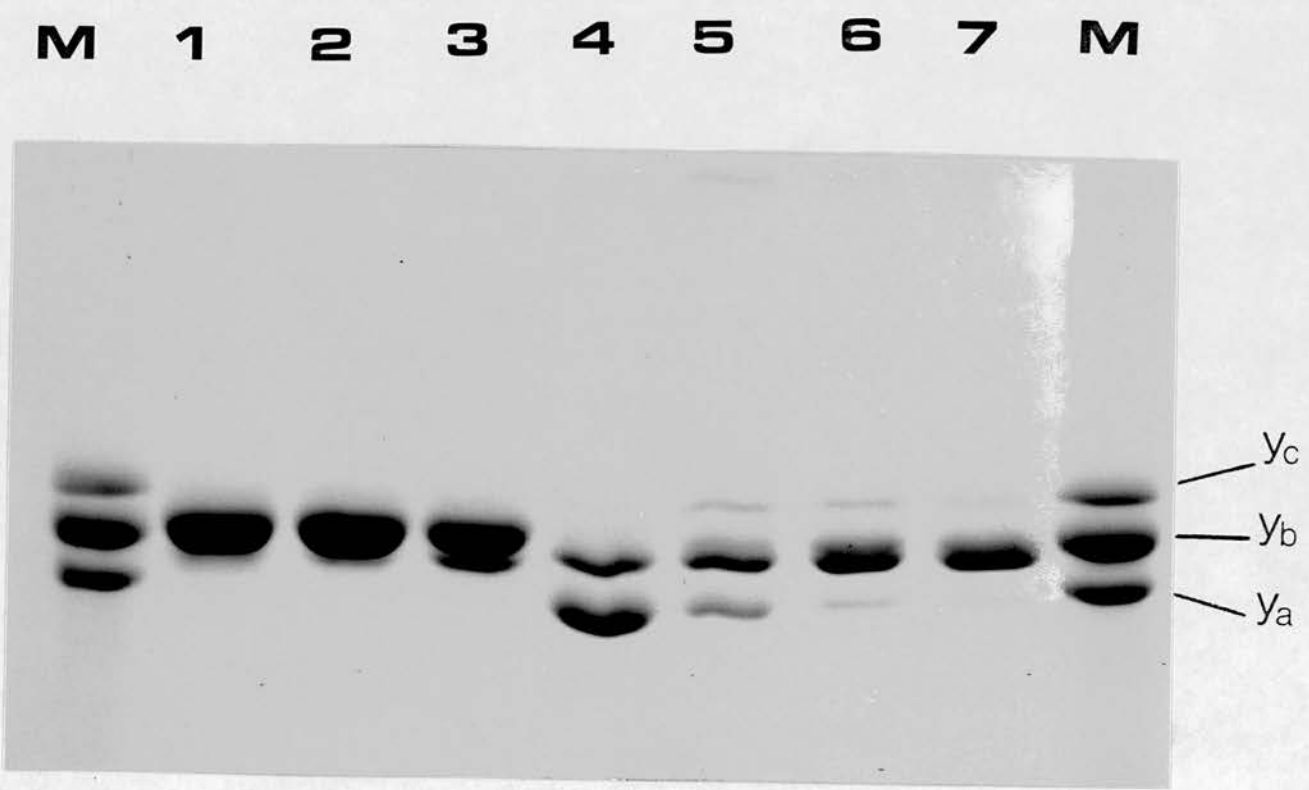
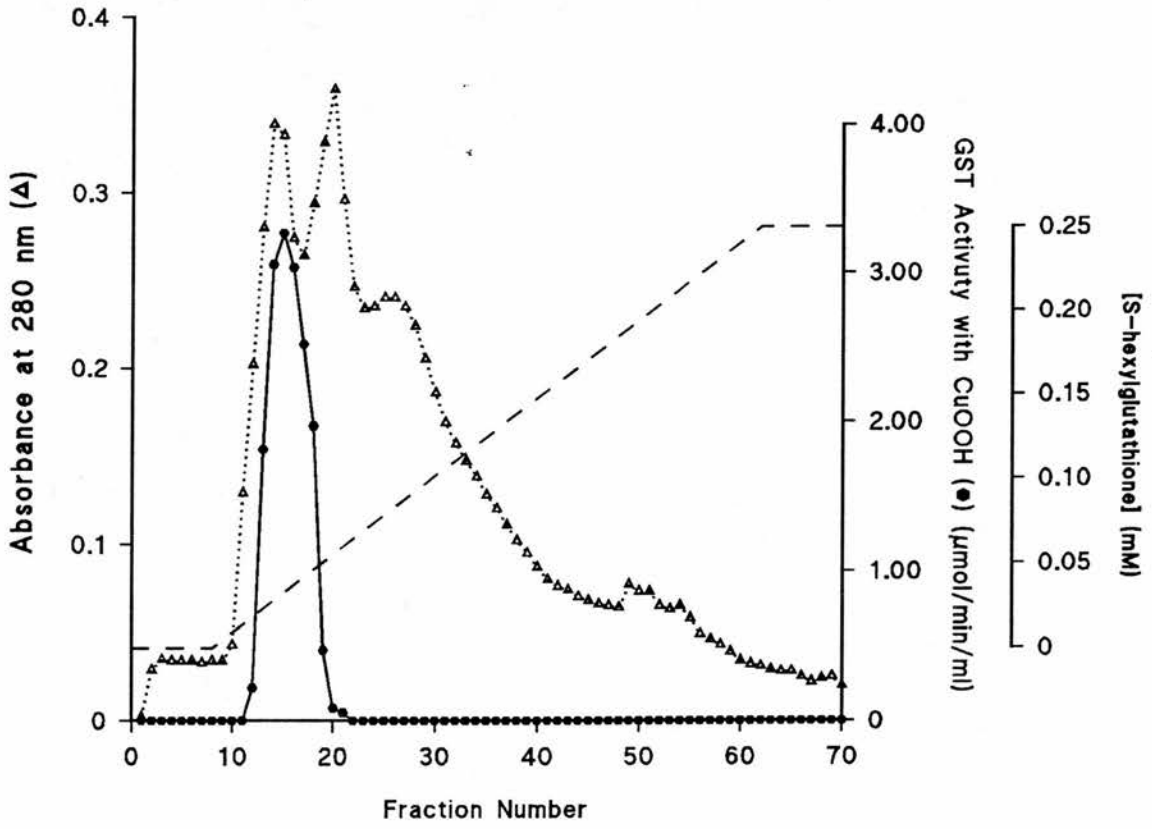

M
1
2
3
4
5
6
7
M
yc
yb
ya

Figure 3.01(H)



Gradient Elution of Bovine Lung GST on S-Hexylglutathione-Sepharose 6B.

Fractions collected during gradient elution of bovine lung GST from S-hexylglutathione-Sepharose 6B were each assayed for activity with cumene hydroperoxide (see "methods"). The values obtained for each fraction were plotted alongside those for protein concentration.

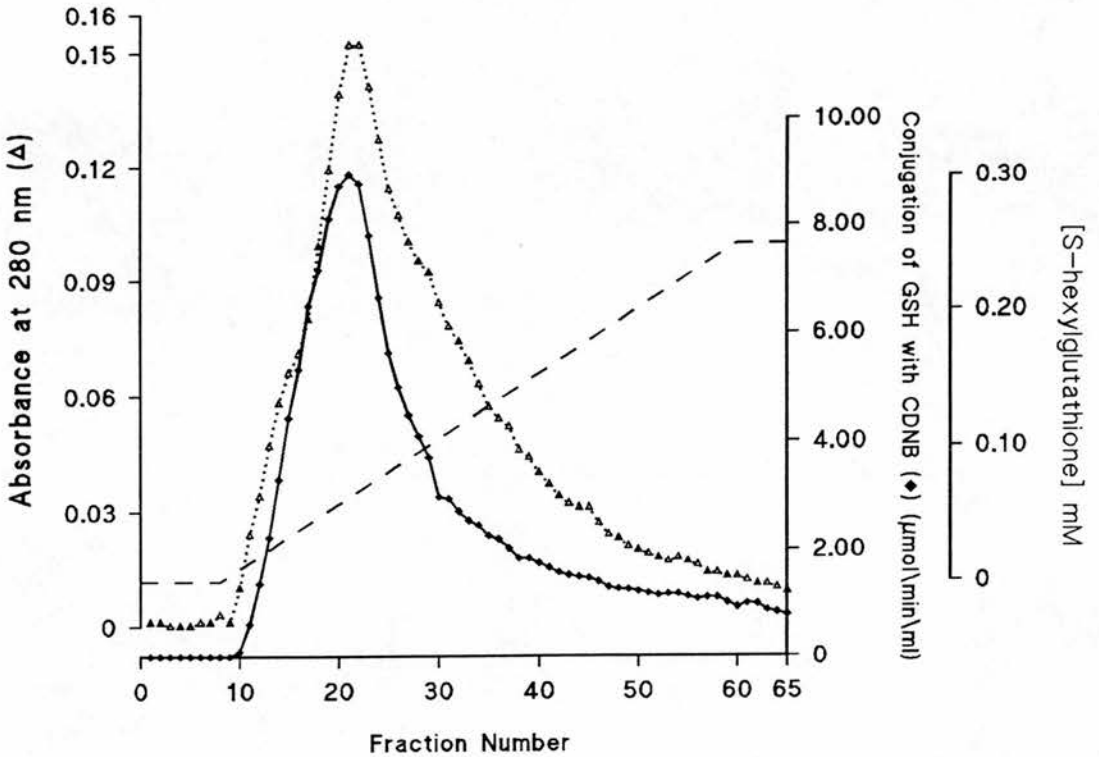
Figure 3.01 (I)

Gradient Elution of Bovine Spleen GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine spleen tissue were prepared (see "methods") and applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The GST isoenzymes bound to this matrix were eluted using a gradient of S-hexylglutathione (0-0.25 mM), and 6.5 ml fractions collected. Each fraction was assayed for GST activity (with CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 15; lane 2, fraction 18; lane 3, fraction 21; lane 4, fraction 24; lane 5, fraction 27; lane 6, fraction 30.

Figure 3.01(I)


Absorbance at 280 nm (Δ)
0.16
0.15
0.12
0.09
0.06
0.03
0
10.00
8.00
6.00
4.00
2.00
0
Conjugation of GSH with CDNB (♦) (μmol\min\ml)
0.30
0.20
0.10
0
[S-hexylglutathione] mM
0
10
20
30
40
50
60
65
Fraction Number

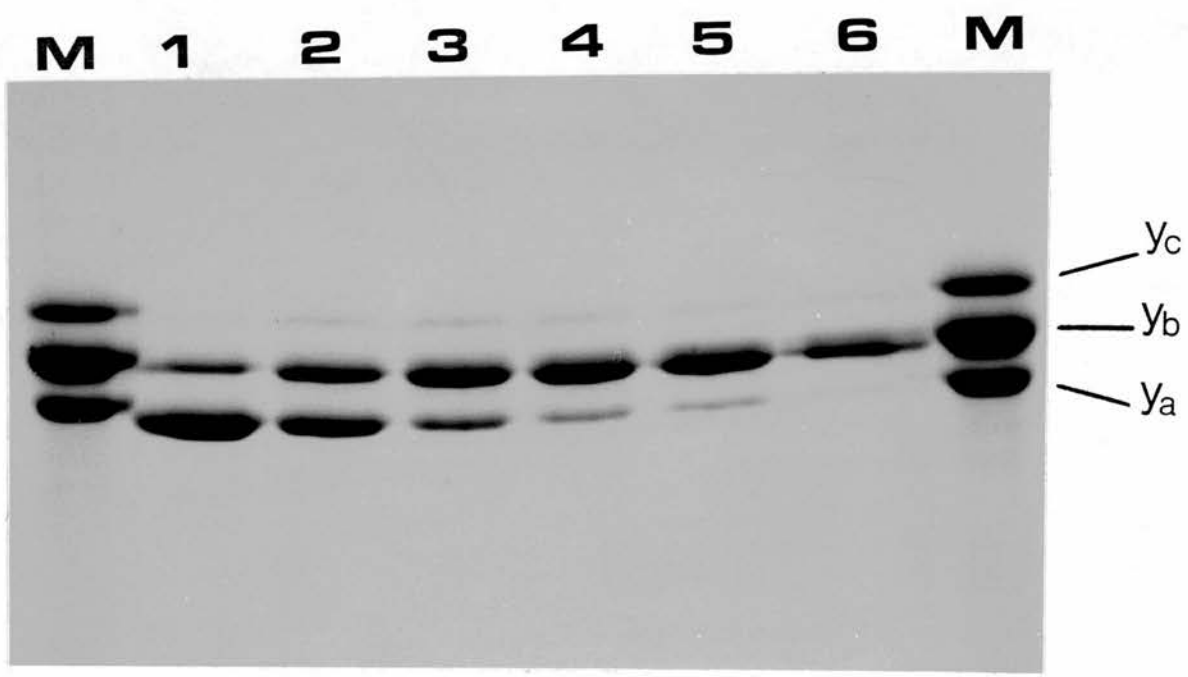

M
1
2
3
4
5
6
M
Yc
Yb
Ya

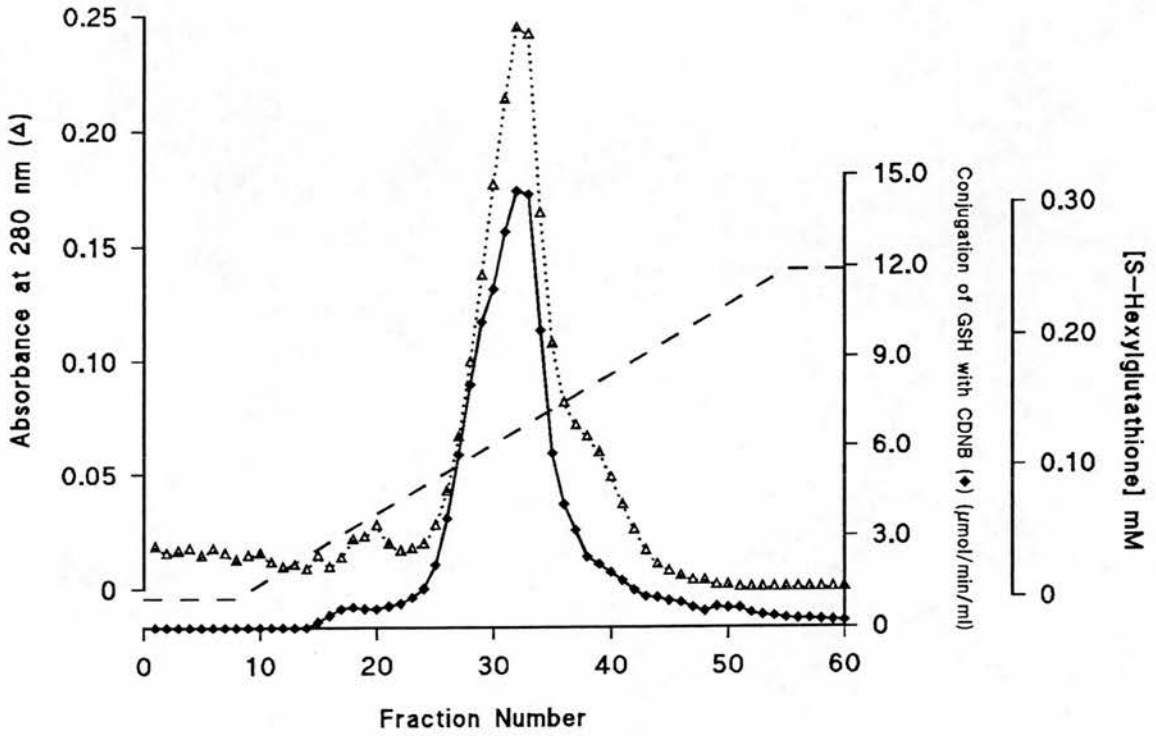
Figure 3.01(J)

Gradient Elution of Bovine Kidney GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions from bovine kidney were prepared (see "methods") and applied to columns (1.6 x 30 cm) containing S-hexylglutathione-Sepharose 6B. The GST isoenzymes bound to this affinity matrix were eluted using a gradient of S-hexylglutathione (0-0.25 mM), and collected into 6.5 ml fractions. Each fraction was assayed for GST activity (with CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 20; lane 2, fraction 27; lane 3, fraction 30; lane 4, fraction 32; lane 5, fraction 34; lane 6, fraction 37.

Figure 3.01(J)


Absorbance at 280 nm (Δ)
0.25
0.20
0.15
0.10
0.05
0
15.0
12.0
9.0
6.0
3.0
0
Conjugation of GSH with CDNB (♦) (μmol/min/ml)
0.30
0.20
0.10
0
[S-Hexylglutathione] mM
0
10
20
30
40
50
60
Fraction Number

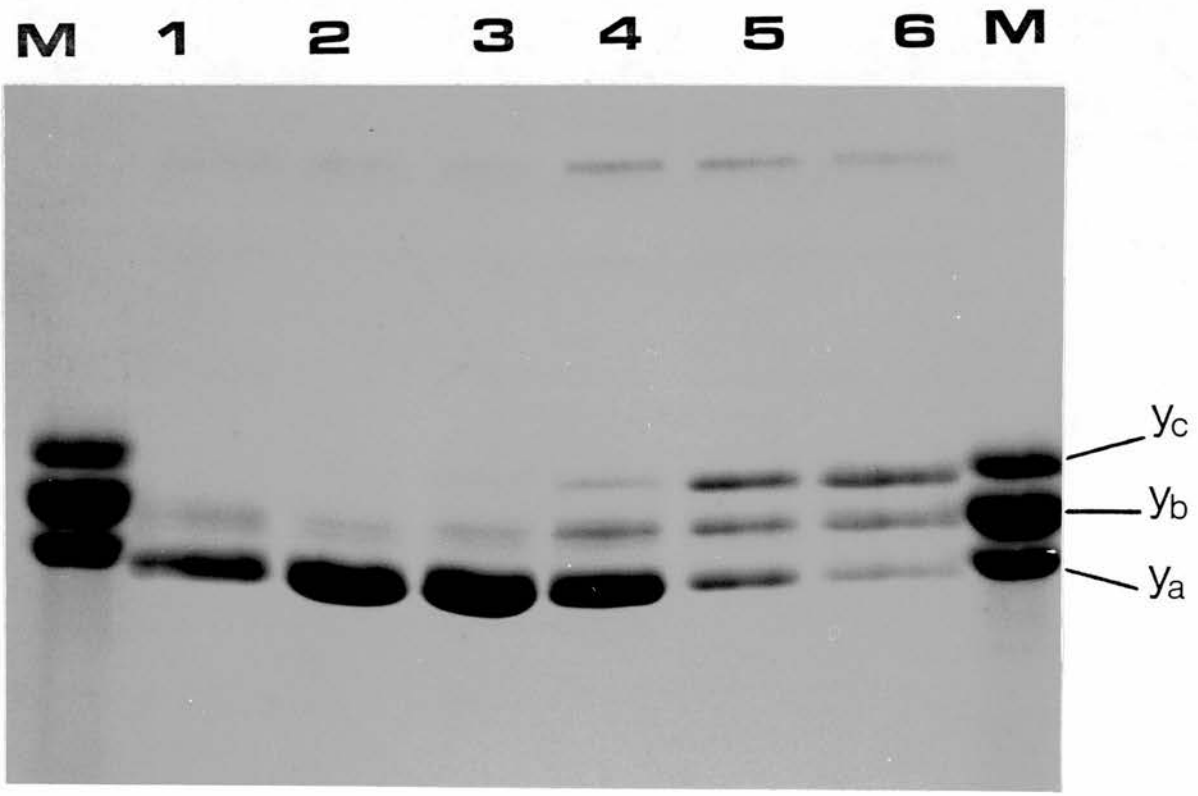

M
1
2
3
4
5
6
M
Yc
Yb
Ya

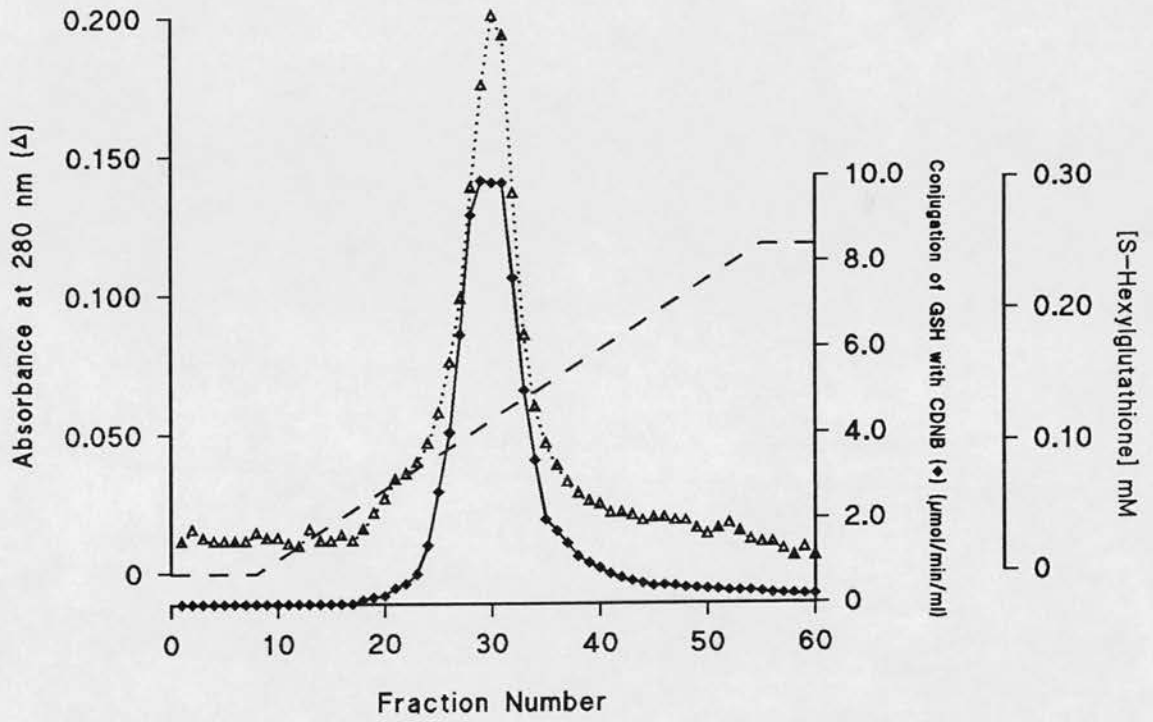
Figure 3.01(K)

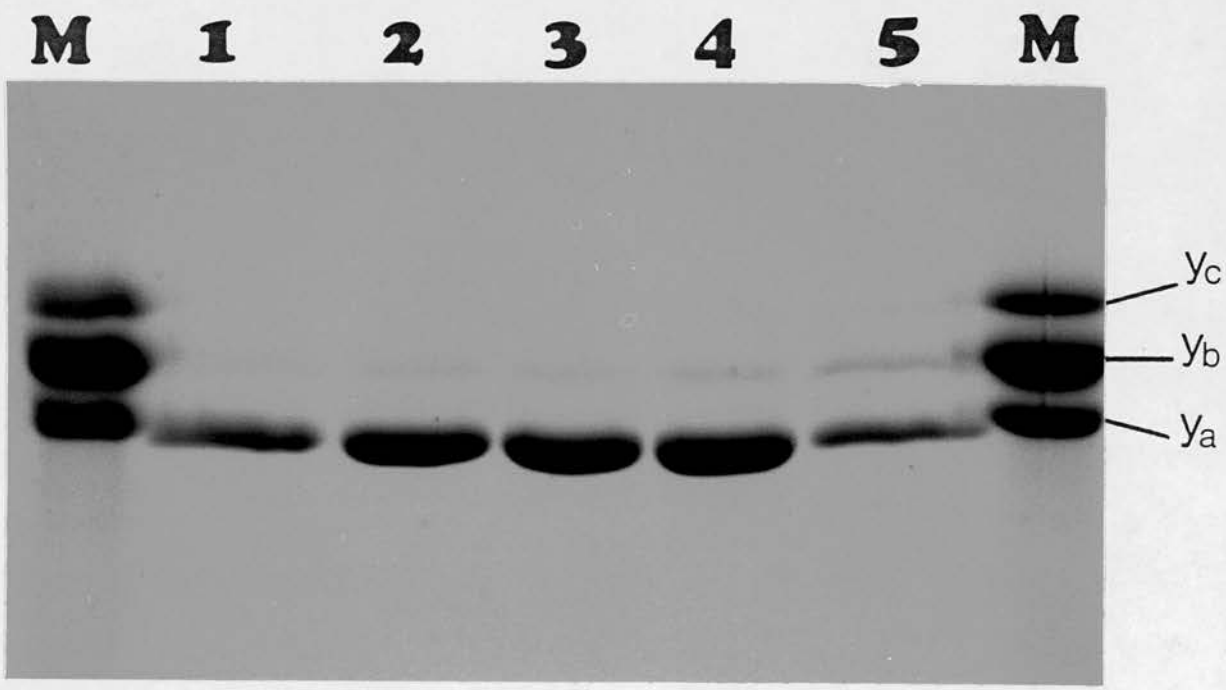
Gradient Elution of Bovine Heart GST on S-Hexylglutathione-Sepharose 6B.

Cytosolic fractions were prepared from bovine heart tissue (as described in "methods") and applied to columns (1.6 x 30 cm) that contained S-hexylglutathione-Sepharose 6B. The GST isoenzymes bound to this matrix were eluted on a gradient of S-hexylglutathione (0-0.25 mM), and collected in 6.5 ml fractions. Each fraction was assayed for GST activity (with CDNB) and the absorbance at 280 nm was measured.

Various fractions were selected for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The gel, shown opposite, was loaded as follows: lanes designated "M" contained rat liver GST isoenzyme mixtures comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 26; lane 2, fraction 28; lane 3, fraction 31; lane 4, fraction 34; lane 5, fraction 37.

Figure 3.01(K)


Absorbance at 280 nm (Δ)
0.200
0.150
0.100
0.050
0
10.0
8.0
6.0
4.0
2.0
0
Conjugation of GSH with CDNB (•) (μmol/min/ml)
0.30
0.20
0.10
0
[S-Hexylglutathione] mM
0
10
20
30
40
50
60
Fraction Number


M
1
2
3
4
5
M
Yc
Yb
Ya

peak 1 contained alpha-class GST since these were the only fractions with significant glutathione peroxidase activity (Fig. 3.01.H). The second peak in the elution profile using CDNB as the substrate consisted of the fast-migrating pi-class GST subunit with similar electrophoretic mobility to the one found in the adrenal cortex (see lane 4, Fig. 3.01.G), with the remaining fractions (fractions 23 onwards) also consisting of the same mu-class GSTs found in the other bovine organs studied (lanes 6 and 7, Fig. 3.01.G).

Several bovine organs did not appear to express alpha-class GSTs, according to affinity chromatography on S-hexG-Ag. Bovine spleen, for example, was found to express only pi- and mu-classes of GST (Fig. 3.01.I). No fractions showed activity with cumene hydroperoxide, consistent with an absence of alpha-class enzymes. The elution profile consisted of one main peak which gradually tailed off as the gradient increased. Fractions selected for SDS/PAGE analysis showed the main peak of activity (fractions 15-21) to consist largely of the pi-class GST subunit discussed above. Later fractions were found to contain mainly the two mu-class GST subunits that are expressed in the other organs studied.

A similar situation was found for both bovine kidney and heart (Figs. 3.01.J and 3.01.K), with the single peak on the elution profiles consisting mainly of the pi-class GST subunit, along with small amounts of the two mu-class GST subunits. Again, none of the fractions collected for both organs showed significant activity with cumene hydroperoxide, confirming the lack of alpha-class GSTs in these organs.

3.02 (A) Analysis of GST Isoenzymes in Bovine Adrenal Cortex that Bind to S-Hexylglutathione-Sepharose 6B (S-hexG-Ag)

Having examined the GST complement of a number of different bovine organs by using gradient elution on S-hexG-Ag, further experiments were performed to characterise the GST isoenzymes in bovine adrenal cortex fully.

(i) Isoelectric Focusing of GSTs from Bovine Adrenal Cortex

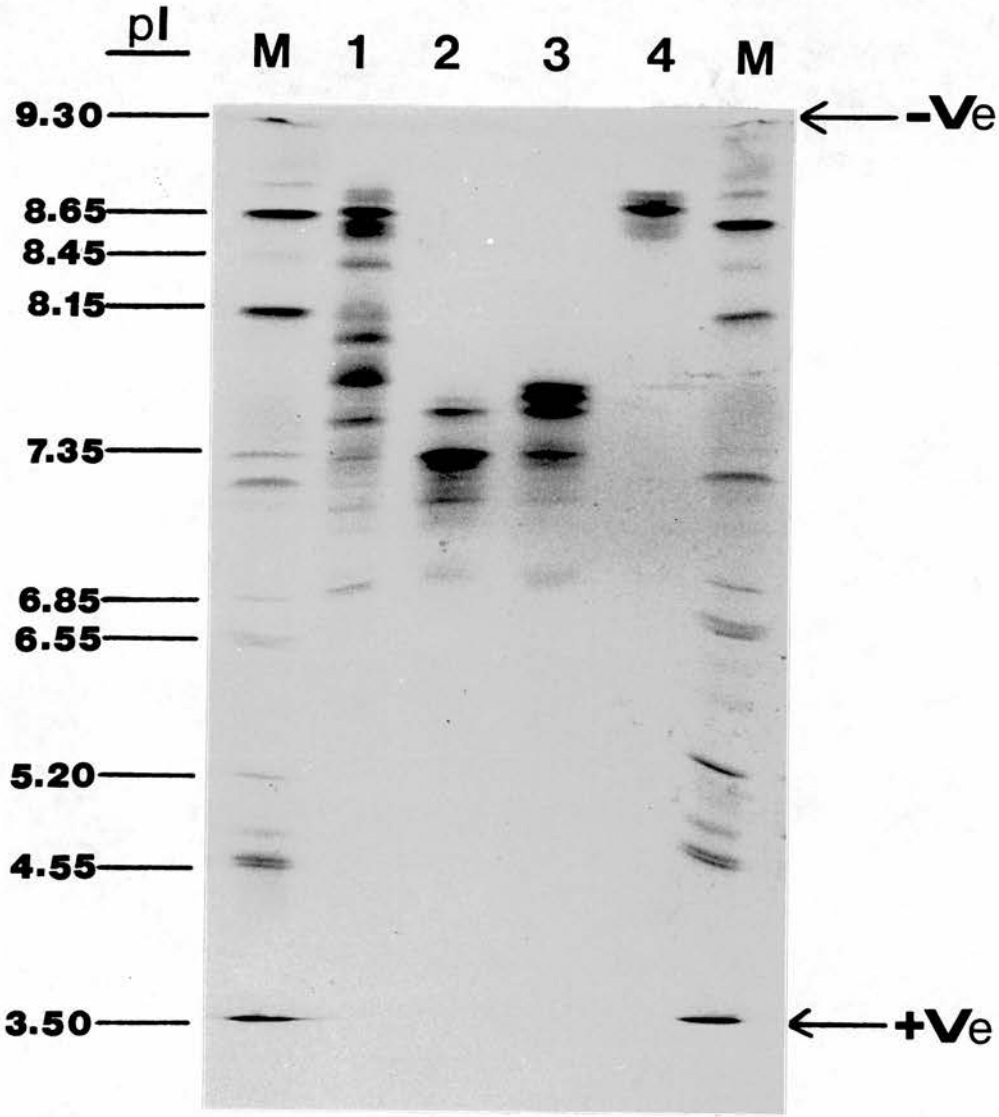
In order to assist the selection of further chromatographic steps to be used to purify individual GST subunits, measurement of the isoelectric points of these proteins was required.

Figure 3.02.A

Analytical Isoelectric Focusing of Bovine Adrenal Cortex GSTs.

Isoelectric focusing was carried out as described (see "methods") using GST isoenzyme pools which had been affinity-purified from bovine adrenal cortex on S-hexylglutathione-Sepharose 6B (see fig. 3.01.A). The gel loadings were as follows: lanes designated "M" contained commercially available standards comprising of proteins with a broad pI range (3.50-9.50), as described in the "methods" section; lane 1, GSTs prepared on a glutathione-Sepharose 6B affinity column; lane 2, fraction 23 (mainly pi-class GST) from S-hexylglutathione-Sepharose 6B affinity chromatography; lane 3, fraction 34 (mainly mu-class GST) from S-hexylglutathione-Sepharose 6B affinity chromatography; lane 4, fraction 13 (mainly alpha-class GST) from S-hexylglutathione-Sepharose 6B affinity chromatography.

Figure **3.02.A**



Such information in the literature is scant, and isoelectric focusing (IEF) was therefore carried out using selected fractions eluted from the S-hexG-Ag affinity column. The fractions selected for IEF corresponded to those containing either mainly alpha-class GST (fraction 13, Fig. 3.01.A), pi-class GST (fraction 23, Fig. 3.01.A) or mu-class GST (fraction 34, Fig. 3.01.A).

Flat-bed IEF was carried out using a broad pH range (3.50 - 9.30) since no information was available on the IEF range of bovine GSTs. The alpha-class GST pool appeared as one main band along with several split bands in the basic region of the gel, indicating an approximate isoelectric point of 8.75 (lane 4, Fig. 3.02.A). Both mu- and pi-class GST pools focussed in a more neutral region of the gel: the pi-class GST pool (lane 2, Fig. 3.02.A) consisted mainly of one intensely-staining band with an approximate isoelectric point of 7.30; the mu-class GST pool (lane 3, Fig. 3.02.A) appeared as three intensely-staining bands with isoelectric points ranging from 7.80 - 7.90.

(ii) Reverse-phase h.p.l.c. of Affinity-purified Alpha-class GST

Those fractions containing alpha-class GSTs from affinity chromatography on S-hexG-Ag were pooled together (fractions 12-18, Fig. 3.01.A) and further purified by reverse-phase h.p.l.c. (Fig. 3.02.B). Two peaks were resolved using this method, each of which was collected and subsequently analysed by SDS/PAGE. The resulting gel (Fig. 3.02.B) revealed peak 1 to correspond to the slower-migrating GST subunit observed initially by SDS/PAGE analysis of the S-hexG-Ag purified pool (designated Ya_1), and peak 2 the faster-migrating GST subunit (designated Ya_3). These purified subunits were used to raise antisera for use in future experiments.

(iii) Anion-exchange Chromatography of Mu/Pi GST Classes

Attempts were made to purify the individual mu-class enzymes using anion-exchange chromatography. Initial application of the mu-class GST pool to mono-Q equilibrated with a buffer adjusted to pH 8.5 did not result in further purification (Fig. 3.02.C). During each chromatographic run one main peak was obtained, suggesting that the 2 subunits apparent on the SDS/PAGE gel of the affinity-purified pool had not been resolved. Subsequent SDS/PAGE analysis of the fractions collected during anion-exchange chromatography confirmed these suspicions (results

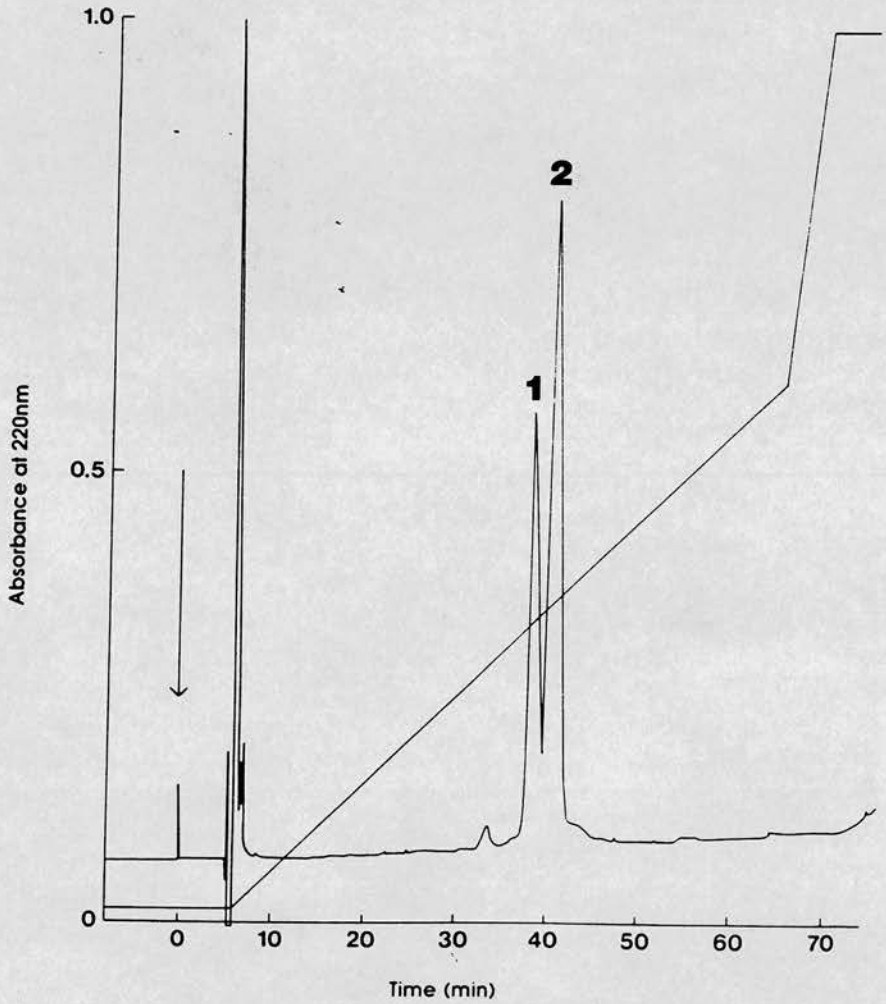
Figure 3.02.B

Reverse-Phase hplc Analysis of Alpha-Class GST from Bovine Adrenal Cortex.

The alpha-class GST isoenzyme pool purified by affinity chromatography on S-hexylglutathione-Sepharose 6B (fig. 3.01.A., fractions 12-18) was examined by reverse-phase hplc. The column employed was a Waters μBondapak C_{18} column (10 μm particle size; column size 0.39 x 30 cm) which was developed at 1 ml/min using a linear 40-58% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 60 min. This was followed by a 58-70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous line; pump A delivered 40% acetonitrile, and pump B 70%.

Each of the 2 peaks obtained was collected and analysed by SDS/PAGE using 12% polyacrylamide resolving gels (see "methods"). The corresponding gel, shown opposite, revealed 2 distinct polypeptides with lane 1 corresponding to peak 1 (Ya_1 subunit) and lane 2 to peak 2 (Ya_3 subunit). The arrow indicates the point of injection of the sample.

Figure **3.02.B**


1.0
0.5
0
Absorbance at 220nm
1
2
0
10
20
30
40
50
60
70
Time (min)

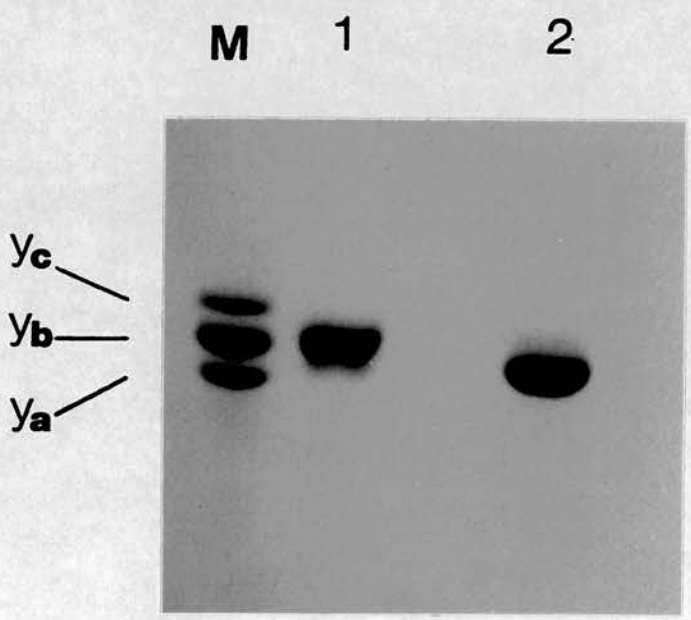
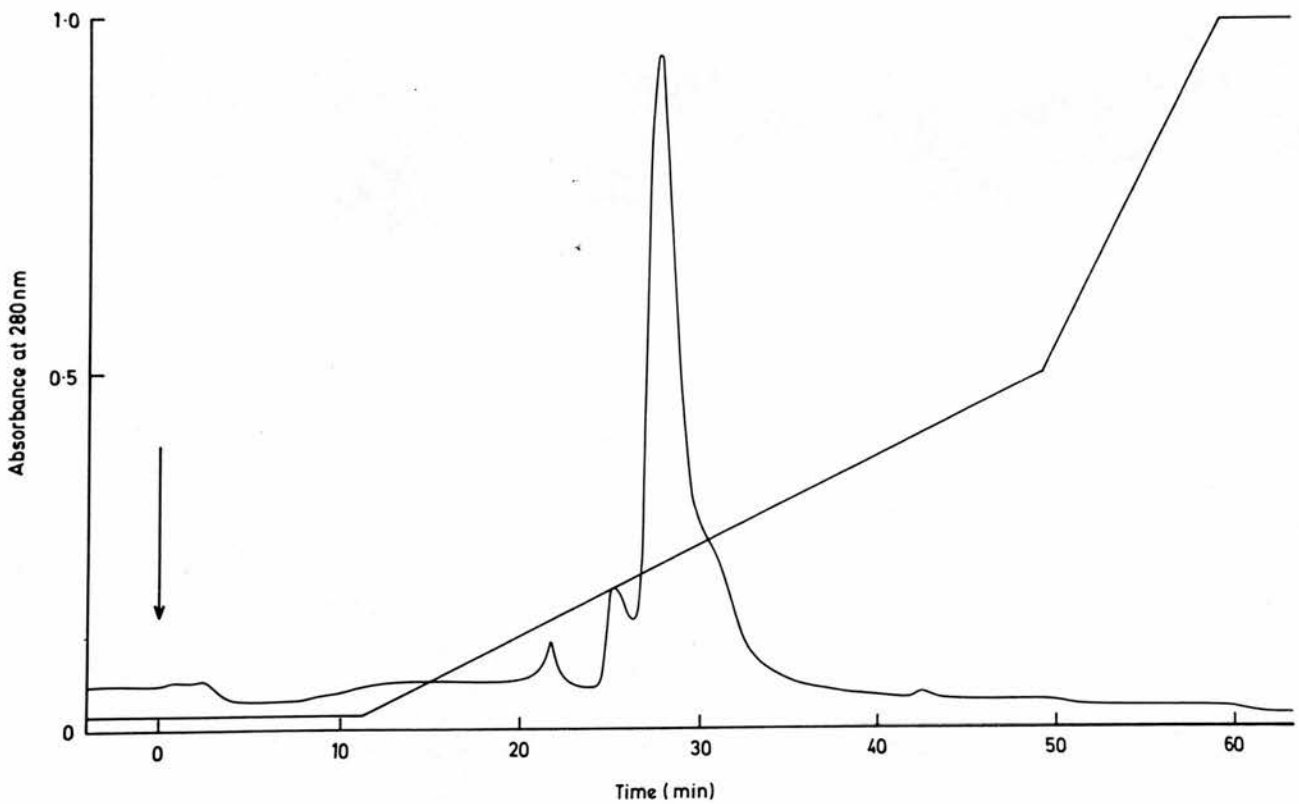

M
1
2
yc
yb
ya

Figure **3.02.c**



Anion-Exchange Chromatography of Mu-Class GST from Bovine Adrenal Cortex.

The mu-class GST isoenzyme pool purified by affinity chromatography on S-hexylglutathione-Sepharose 6B (fig. 3.01.A, fractions 32-40) was characterised further on the Pharmacia FPLC system using the mono-Q anion exchange column (see "methods"). Aliquots of this enzyme pool, which had been equilibrated with 0.25 mM tris buffer (pH 8.5) containing 2 mM 2-mercaptoethanol, were applied to the column, which had also been equilibrated with the same buffer. The limit buffer contained 0.5 mM NaCl, and the eluate was monitored continuously at 280 nm. A flow-rate of 0.75 ml/min was used, and 0.75 ml fractions were collected. Following application of the sample (as indicated by the arrow) and a subsequent loading time of 10 min, a linear 0-50 % salt gradient was used to resolve the bound material.

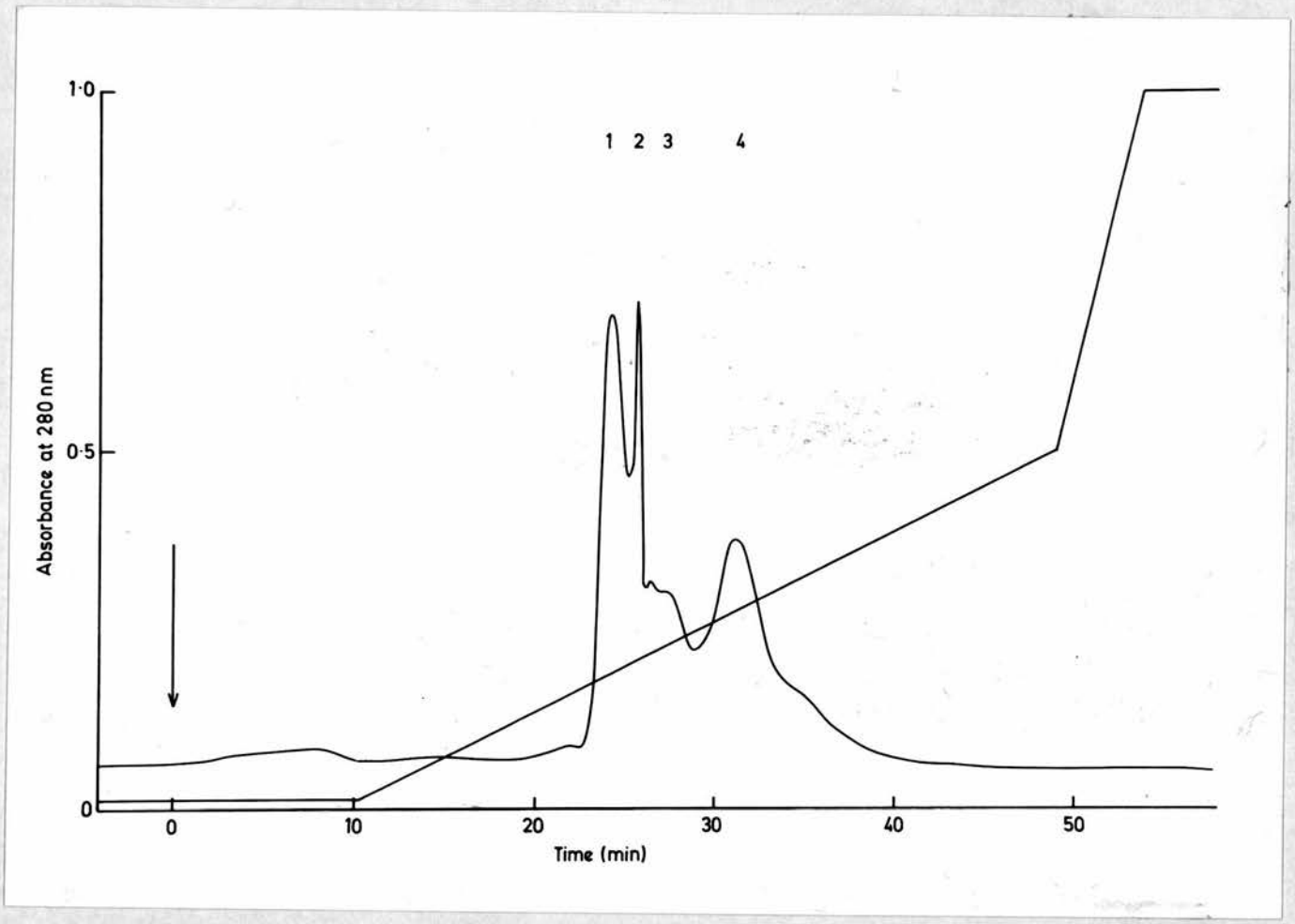
Figure 3.02.D

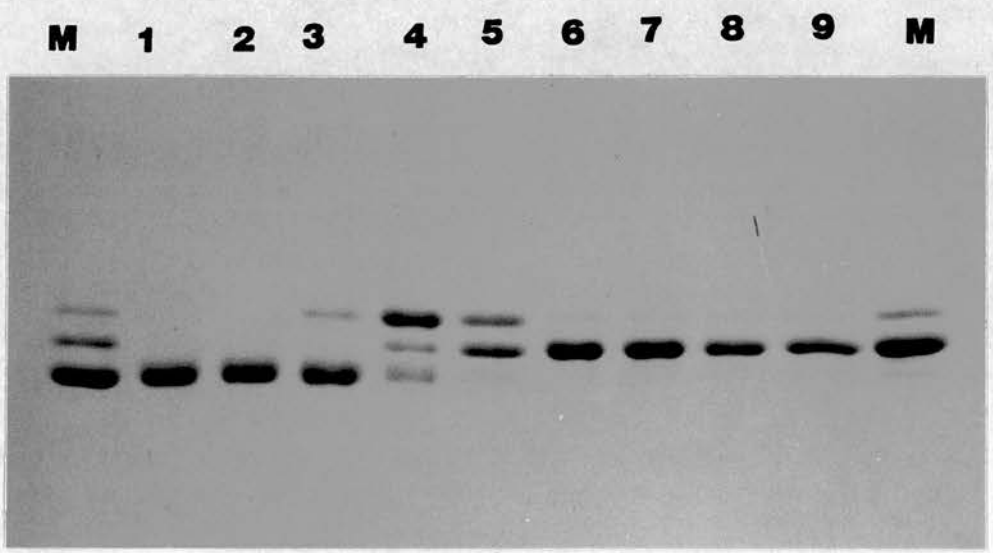
Anion-Exchange Chromatography of Mu and Pi Class GST from Bovine Adrenal Cortex.

The GST isoenzyme pool comprising both pi and mu classes from bovine adrenal cortex (fractions 22-32, Fig. 3.01.A), which had been purified by affinity chromatography on S-hexylglutathione-Sepharose 6B, was further purified by anion-exchange chromatography on the Pharmacia FPLC system using the mono-Q column (see "methods"). Both the enzyme pool and anion-exchange column were equilibrated with 0.25 mM Tris/HCl buffer (pH 8.9) containing 2 mM 2-mercaptoethanol prior to each chromatographic run. The limit buffer contained 0.5 M NaCl, and the eluate was monitored at 280 nm. The flow-rate used was 0.75 ml/min and 0.75 ml fractions were collected throughout the duration of each chromatographic run. Following application of the sample (as indicated by the arrow) and a subsequent 10 min loading time, the bound isoenzymes were resolved over 40 min using a linear 0-50 % salt gradient. During each run, 4 main peaks were obtained as indicated.

Certain fractions were selected from this chromatography step for SDS/PAGE analysis using 12% polyacrylamide resolving gels. The corresponding gel, shown opposite, consisted of the following loadings: lane M_1, affinity-purified pi-class marker GST (fig. 3.01.A, fraction 23); lane 1, fraction 25 from anion-exchange chromatography; lane 2, fraction 26; lane 3, fraction 27; lane 4, fraction 28; lane 5, fraction 29; lane 6, fraction 31; lane 7, fraction 32; lane 8, fraction 33; lane 9, fraction 34; lane M_2, affinity-purified mu-class GST marker (fig. 3.01.A, fraction 34).

Figure **3.02.D**


1·0
0·5
0
Absorbance at 280 nm
1 2 3 4
0
10
20
30
40
50
Time (min)


M 1 2 3 4 5 6 7 8 9 M

not shown), and further attempts were made using the same technique but with the buffer adjusted to pH 8.9. Those fractions containing both mu- and pi- class GSTs were combined (fractions 22-32, Fig. 3.01.A) and, following application to the anion-exchange column, four main peaks were resolved (fig. 3.02.D). Various fractions were selected across the elution profile from this column and analysed by SDS/PAGE: peaks 1 and 2 consisted of the pi-class GST subunit (lanes 1 and 2, Fig. 3.02.D); peak 3 consisted of almost equimolar amounts of the two mu-class subunits (lane 5, Fig. 3.02.D); peak 4 consisted of both mu-class subunits (although with the slow-migrating subunit at a reduced level) in the ascending part of the peak (lane 6, Fig. 3.02.D), with the faster-migrating subunit in the descending part (lanes 7, 8 and 9; Fig. 3.02.D).

(iv) Reverse-phase h.p.l.c. Analysis of Pi-/Mu-class GST Isoenzymes Purified by Anion-exchange Chromatography

Samples from each of the peak fractions collected during anion-exchange chromatography were applied to a reverse-phase h.p.l.c. column. The sequential application of an aliquot from each peak enabled the resolution of four main peaks by reverse-phase h.p.l.c. (Fig. 3.02.E (i-iv)). Application of either peak 1 or 2 from the anion-exchange run (i.e. homogeneous pi-class GST subunits) resulted in the resolution of two peaks during reverse-phase h.p.l.c. (Fig. 3.02.E (i)) named peaks a and b; subjection of peak 3 from anion-exchange chromatography to reverse-phase hplc resulted in the appearance of the same two peaks but with the addition of another peak higher up the elution gradient named peak d (Fig. 3.02.E (ii)); peak 4 from anion-exchange chromatography resulted in the resolution of two peaks on reverse-phase h.p.l.c. named peaks c and d (Fig. 3.02.E (iii)); finally, simultaneous application of equal amounts of peaks 1 and 4 from anion-exchange chromatography to reverse-phase h.p.l.c. resulted in the resolution of all four peaks (Fig. 3.02.E (iv)). Thus, the mu- and pi-class GST fractions resolved by anion-exchange chromatography could be further resolved into four main peaks (a-d) by reverse-phase h.p.l.c.

Each of the four peaks resolved during reverse-phase h.p.l.c. were collected and analysed by SDS/PAGE (Fig. 3.02.F). Peaks a and b were found to correspond to pi-class GST subunits of Mr 24 900 and Mr 25 100 respectively (lanes 2 and 3, Fig. 3.02.F); peak c corresponded to a

Figure 3.02.E (i-iv)

Reverse-Phase hplc Analysis of Pi and Mu Class GST Isoenzymes Purified by Anion-Exchange Chromatography.

Fractions collected during anion-exchange chromatography of affinity-purified pi and mu class GST from bovine adrenal cortex were sequentially applied to a Waters reverse-phase hplc system. A Waters μBondapak C_{18} column was used (10 μm particle size; column size 0.39 x 30 cm) which was developed at 1 ml/min using a linear 40-58% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 60 min. This was followed by a 58-70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous line; pump A delivered 40% acetonitrile, and pump B 70%.

Four sequential chromatography runs were carried out using various fractions collected during the previous anion-exchange step (fig. 3.02.D): run (i) used an aliquot from peak 1 (lanes 1 and 2 in the corresponding gel); run (ii) used an aliquot from peak 3 (lane 5 in the corresponding gel); run (iii) used an aliquot from peak 4 (lanes 8 and 9 in the corresponding gel); and run (iv) consisted of equal amounts of the samples applied during runs (i) and (iii). The arrows indicate the point of application of each sample.

Figure **3.02.E**

i

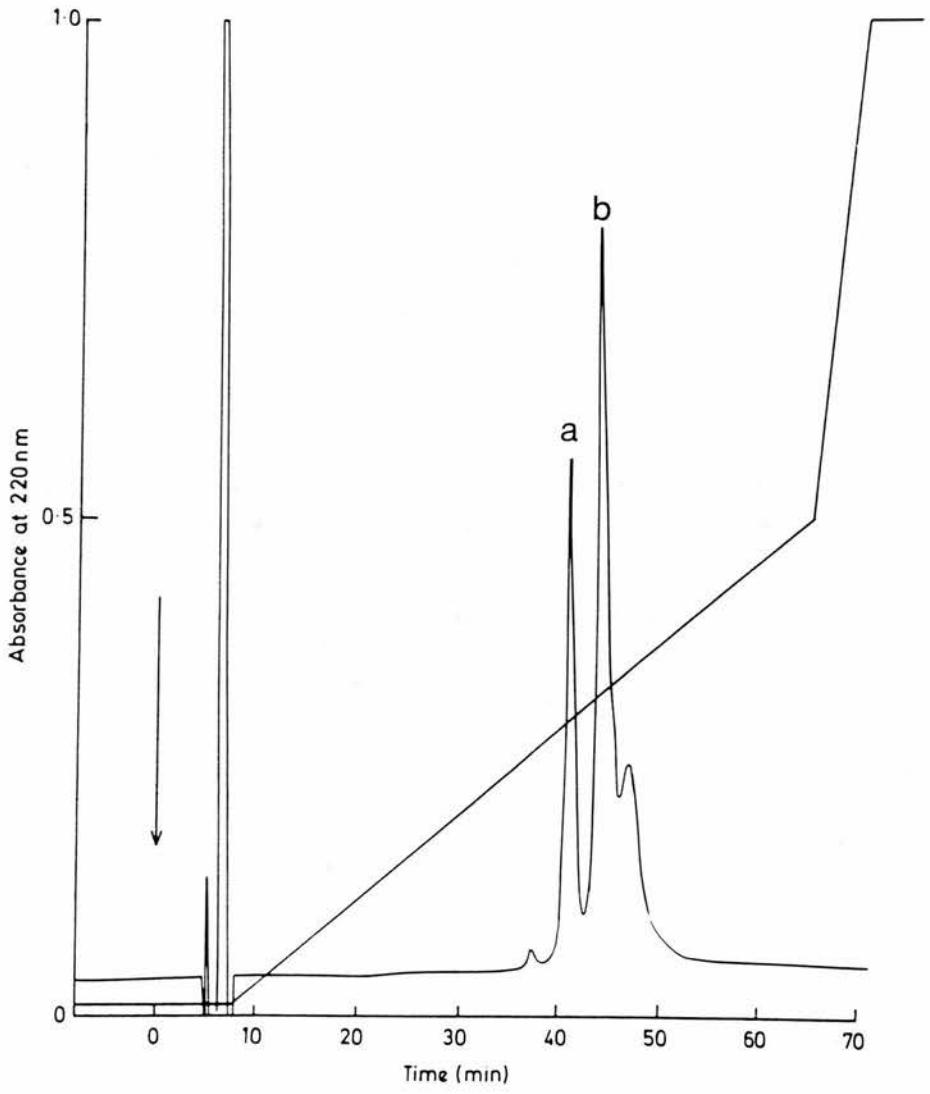


Figure **3.02.E**

ii

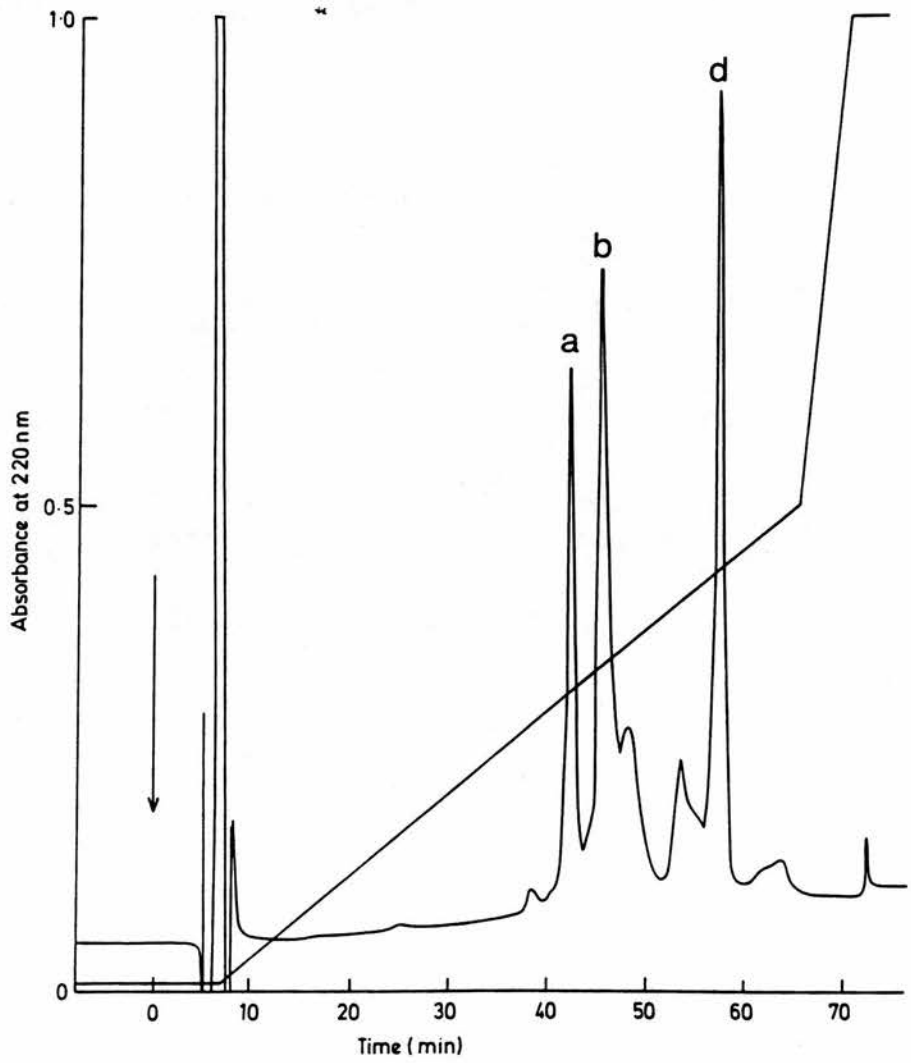


Figure **3.02.E**

iii

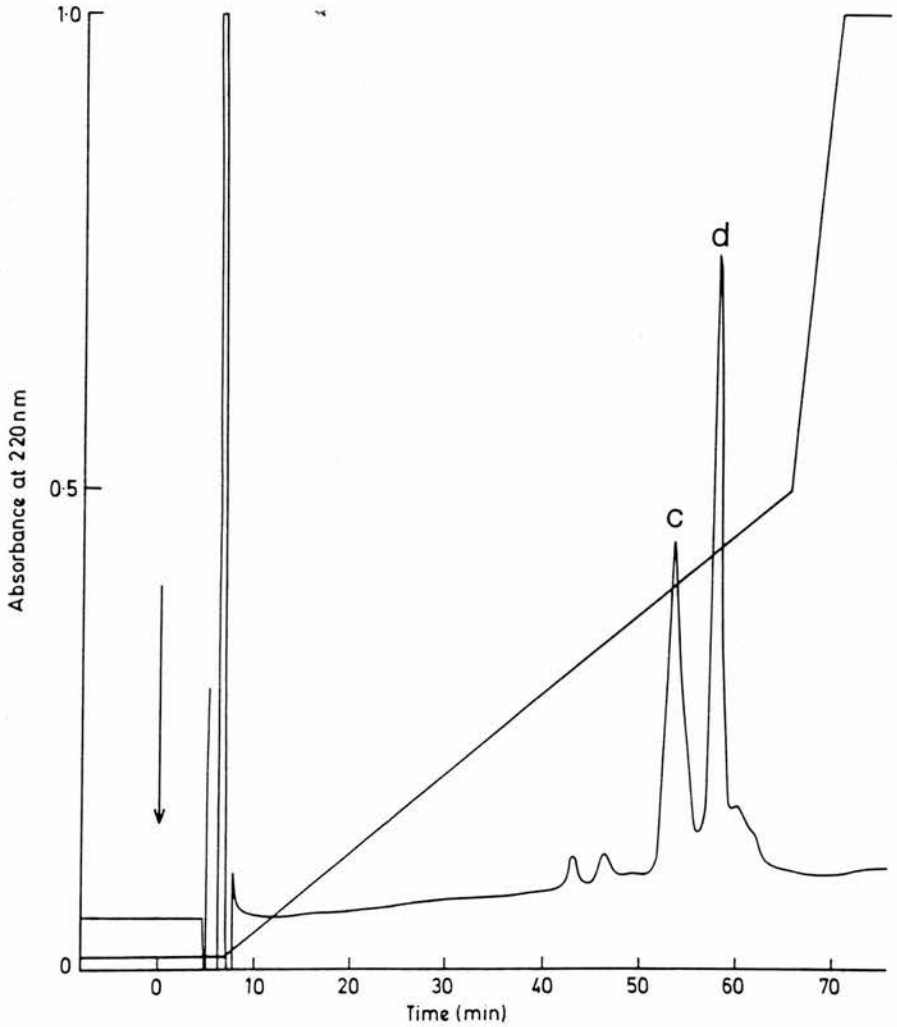


Figure **3.02.E**

iv

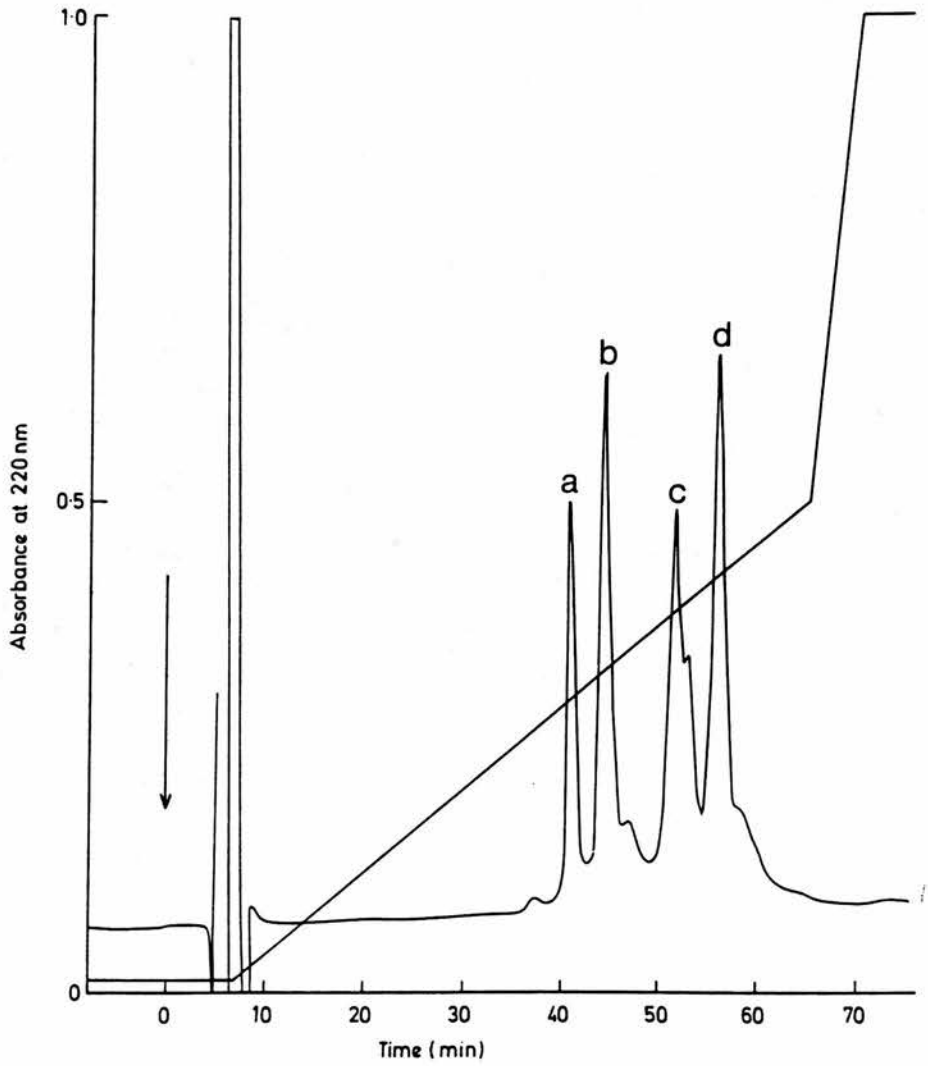
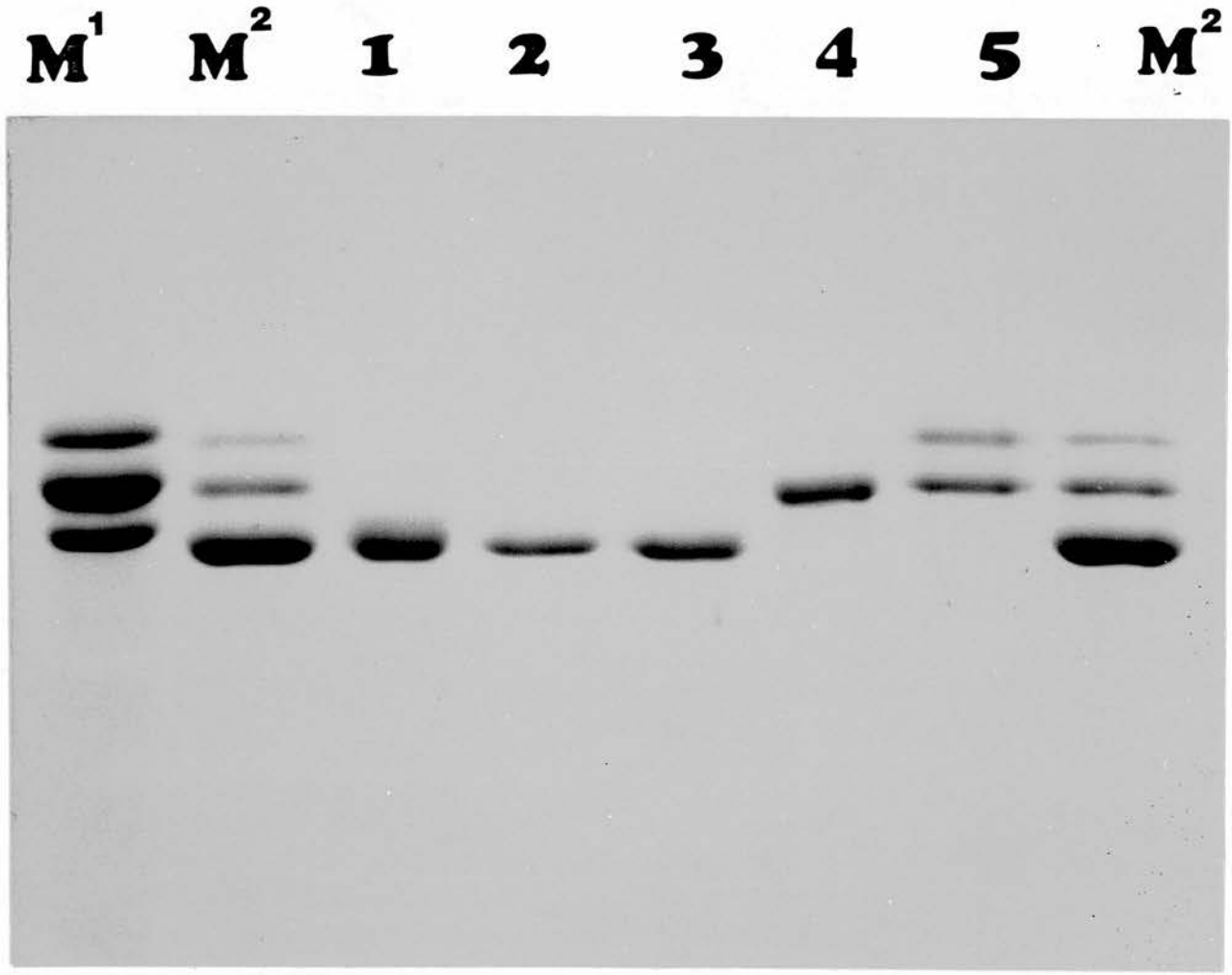


Figure 3.02.F



Electrophoretic Analysis of Bovine Adrenal Cortex Mu and Pi Class GST Subunits Purified on Reverse-Phase hplc.

12% Polyacrylamide resolving gels were used to analyse the GST subunits purified during reverse-phase hplc of partially purified GST isoenzyme pools. Each of the peaks named a-d on profiles i-iv of figure 3.02.E were collected and loaded on the gel as follows: the lane designated "M^{1}" contained a rat GST isoenzyme mixture comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lanes designated "M^{2}" contained the mu/pi class GST pool purified on S-hexylglutathione-Sepharose 6B (fig. 3.01.A, fraction 27); lane 1, "Yf-type" subunit purified by anion-exchange chromatography (fig. 3.02.D, peak 1, lane 1 of gel); lane 2, peak a of fig. 3.02.E (i)-(iii); lane 3, peak b of fig. 3.02.E (i)-(iii); lane 4, peak c of fig. 3.02.E (iii)/(iv); lane 5, peak d of fig 3.02.E (iii)/(iv).

mu-class GST subunit of Mr 26 000 (lane 4, Fig. 3.02.F); peak d corresponded to two mu-class GST subunits of almost equimolar amounts of Mr 26 100 and Mr 27 300.

3.02

(B) Characterisation of Bovine Adrenal Cortex Alpha-Class GSTs that Fail to Bind to S-Hexylglutathione-Sepharose 6B (S-hexG-Ag)

Analysis of the flow-through fraction from the S-hexG-Ag column, following application of bovine adrenal cortex cytosol, revealed that over one-third of the GST activity towards CDNB did not bind to the affinity matrix (Table 3.02.A). Therefore, this fraction was subsequently applied to a second affinity column consisting of glutathione-Sepharose 6B (GSH-Ag) [Fig. 3.02.G]. The majority of the GST activity towards CDNB that failed to bind to S-hexG-Ag was retained by GSH-Ag and was eluted in a single step with 40 mM GSH. The fractions obtained from GSH-Ag were examined as described above.

(i) Enzymic Activity

Following application of the adrenal S-hexG-Ag flow-through to GSH-Ag, the use of 40 mM GSH to develop the second affinity column resulted in the elution of a single large GST-containing peak (Fig. 3.02.H(i)). This peak also displayed relatively high glutathione peroxidase activity, indicative of this peak containing alpha-class GSTs (Fig. 3.02.H(ii)). Fractions 11-14 were pooled and subjected to further enzymic characterisation using other model GST substrates. The results of these assays are included in Table 3.02.B, which also illustrates activities obtained with the same substrates at different stages of affinity chromatography on both S-hexG-Ag and GSH-Ag. Enzyme activities using CDNB revealed that about 35% of the total CDNB activity remained in the flow-through from the S-hexG-Ag column, but that this activity was greatly reduced in the flow-through fraction following application to GSH-Ag. A similar situation was found using cumene hydroperoxide, Δ^5 androstene-3,17-dione and 4-hydroxynon-2-enal as substrates. These substrates all show high activities with alpha-class GST enzymes, again suggesting that the GST(s) purified on GSH-Ag belong to the alpha-class. The peroxidase activity remaining in the flow-through from the second column can be attributed to the selenium-dependent enzyme (GPx),

Table 3.02(A)

GST ACTIVITY IN ADRENAL CORTEX CYTOSOLS BEFORE/AFTER AFFINITY CHROMATOGRAPHY ON S-HEXYLGLUTATHIONE-SEPHAROSE 6B.

SAMPLE	GST Activity with CDNB (μmol/min/mg protein)
Pre-column Cytosol	0.24
Column Flow-through	0.09

Figure 3.02(G)

ALTERNATIVE PURIFICATION OF GST ISOENZYMES ON GLUTATHIONE–SEPHAROSE 6B.

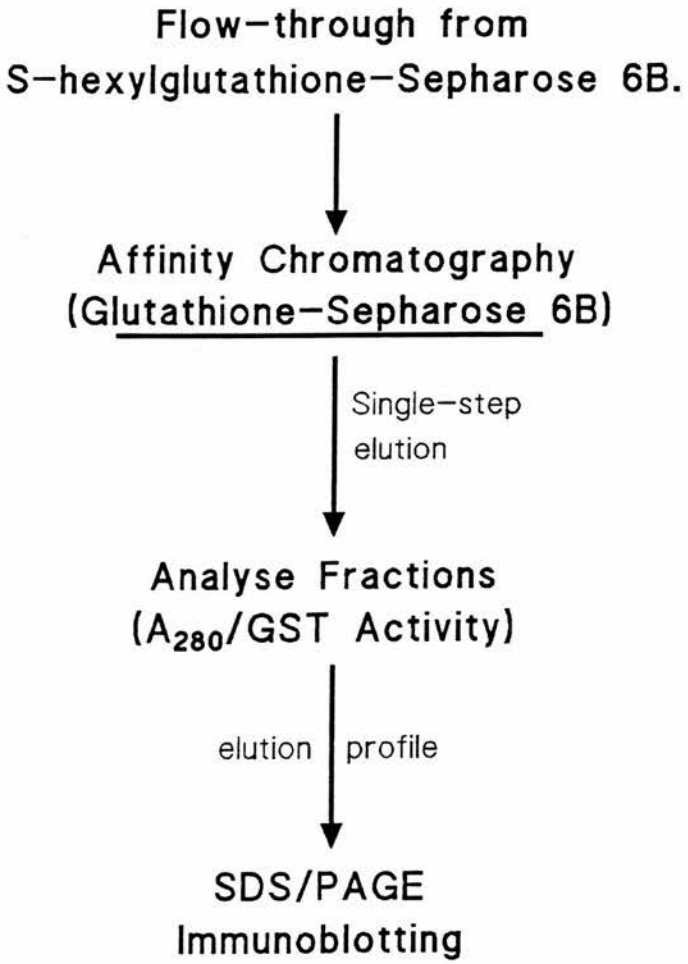


Figure 3.02.H (i/ii)

Single-Step Elution of Bovine Adrenal Cortex GST on Glutathione-Sepharose 6B.

The flow-through fraction from a column containing S-hexylglutathione-Sepharose 6B, following application of bovine adrenal cortex cytosol, was reapplied to a column containing glutathione-Sepharose 6B. The GST isoenzymes bound to this column were eluted using a solution of 40 mM GSH and collected into 6.5 ml fractions. Each fraction was assayed for both protein concentration (absorbance at 280 nm) and GST activity (using both CDNB and CuOOH as substrates).

Figure 3.02.H(i)

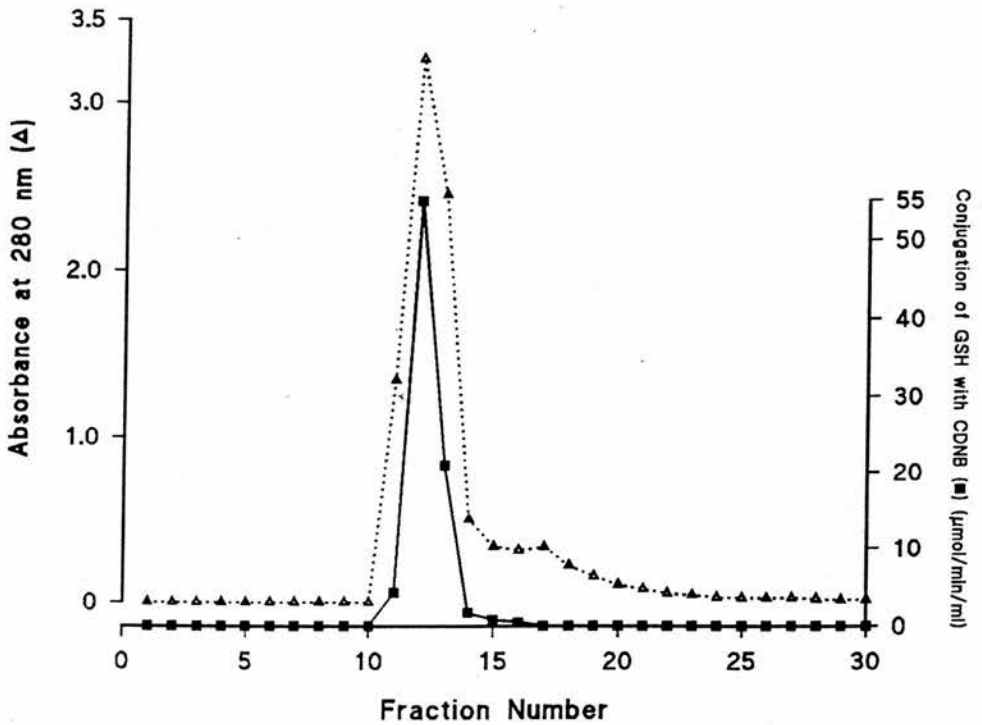
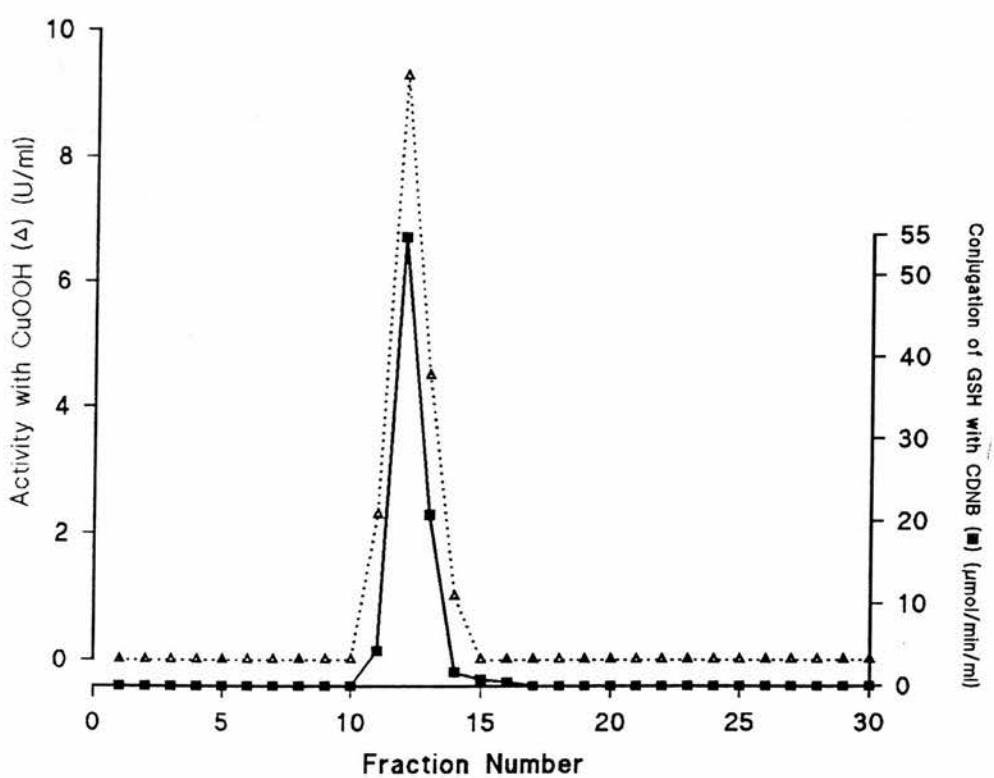


Figure 3.02.H(ii)



which shows activity with cumene hydroperoxide but does not bind to either affinity matrix employed in this thesis.

(ii) SDS/PAGE; Immunoblotting

The different steps used in the purification of bovine adrenal cortex GSTs were studied by analysis of the flow-through fractions at each stage using SDS/PAGE (Fig. 3.02.I(i)). Pre-column bovine adrenal cortex cytosol (lane 1) was found to contain an abundant protein(s) with a similar electrophoretic mobility to rat GST Ya, Yb and Yc subunits, which was also observed in the flow-through fractions from the S-hexG-Ag column (lane 2). However, the material which failed to bind to the GSH-Ag column (lane 3), following application to S-hexG-Ag, did not appear to show the corresponding protein bands. This observation is consistent with the enzyme assay data which suggested the GSH-Ag column had removed these subunits from the S-hexG-Ag flow-through. SDS/PAGE analysis of the GST pool (fractions 11-14 eluted from GSH-Ag, Fig. 3.02.H(i/ii)) revealed this to consist of two distinct bands of unequal staining intensity (lanes 4 and 5, Fig. 3.02.I): a more densely-staining, faster-migrating band of Mr 25 900, and a slower-migrating, less densely-staining band of Mr 26 500. The Mr 25 900 band represents a GST polypeptide which appears particularly abundant in adrenal cortex cytosol, and is estimated to comprise 1.3% of the cytosolic protein in this organ. The GST pool purified on S-hexG-Ag (lane 6) was included in the gel for comparison and shows the two GST subunits described previously: Ya_1 (Mr 27 000) and Ya_3 (Mr 25 900).

Immunoblotting at different stages of affinity chromatography was carried out using antisera raised separately against the purified subunits, Ya_1 and Ya_3, described earlier in this chapter (Fig. 3.02.B). The Western blot employing the anti(Ya_1) antiserum revealed cross-reactivity with certain proteins in the pre-column cytosol (Fig. 3.02.I(ii), blot A, lane 1), with a markedly diminished cross-reactivity in the S-hexG-Ag column flow-through (lane 2) and no cross-reactivity in the flow-through from the GSH-Ag column (lane 3). The alpha-class GST pool purified on GSH-Ag (lanes 4 and 9) and S-hexG-Ag (lanes 5 and 8) showed marked cross-reactivity with this antibody, although, as expected, the other GST classes purified on S-hexG-Ag (fractions 23 and 34, Fig. 3.01A) showed much less cross-reactivity. Immunoblotting of the same samples using

Figure 3.02.I

(i) Electrophoretic Analysis of Bovine Adrenal Cortex Cytosol Preparations at Different Stages of Purification.

Cytosol from bovine adrenal cortex tissue was analysed by SDS/PAGE at different stages of purification by affinity chromatography (60 μg protein per lane). In addition, purified enzymes at each stage were also included. The gel loadings were as follows: lane "M" contained a rat liver GST isoenzyme mixture comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, unfractionated bovine adrenal cortex cytosol; lane 2, flow-through fraction from the S-hexylglutathione-Sepharose 6B column; lane 3, flow-through from the glutathione-Sepharose 6B column; lanes 4 and 5, GST isoenzyme pool purified on glutathione-Sepharose 6B; lane 6, alpha-class GST isoenzyme pool purified on S-hexylglutathione-Sepharose 6B (see fig 3.01.A).

Figure **3.02.I**

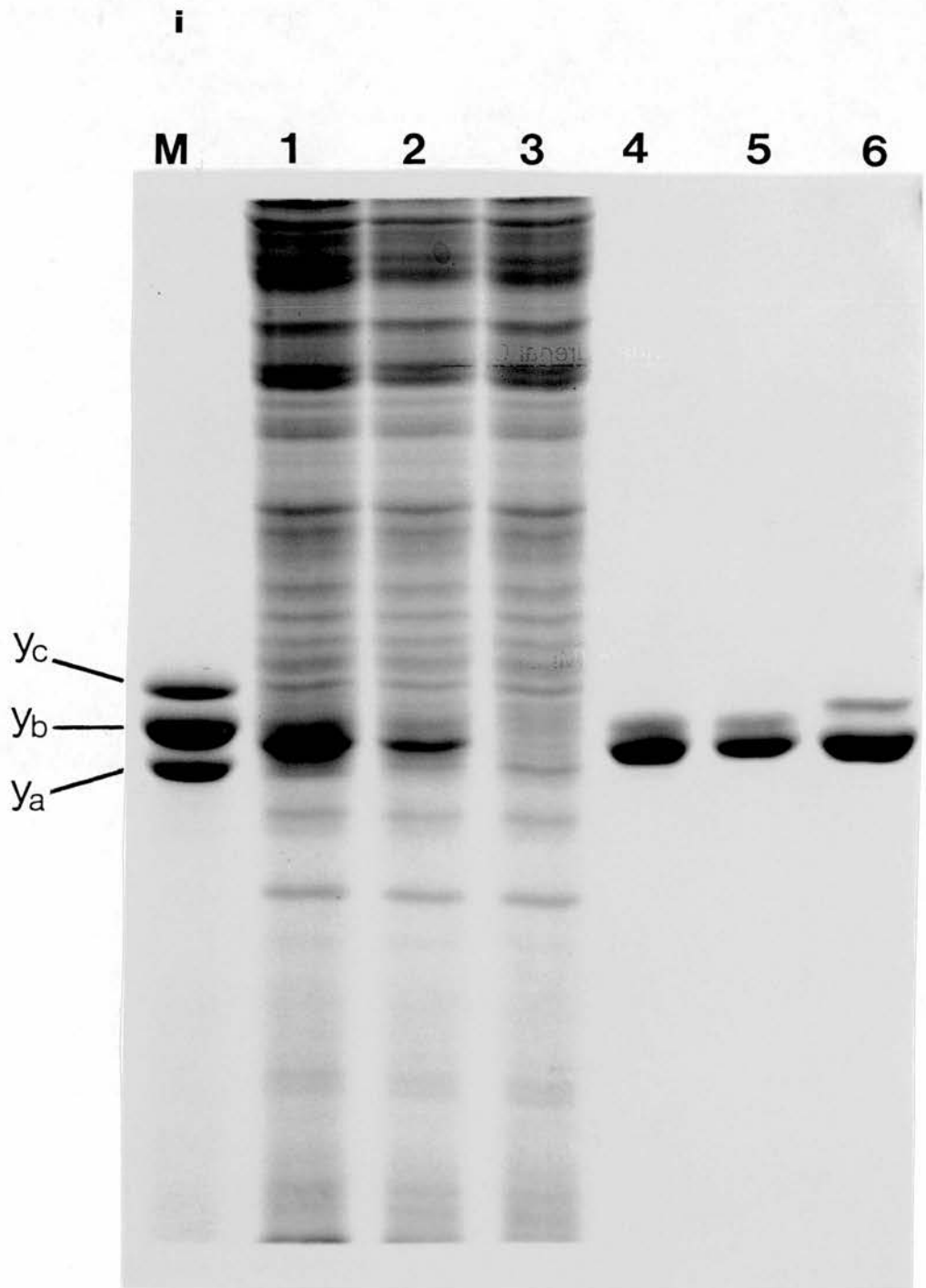


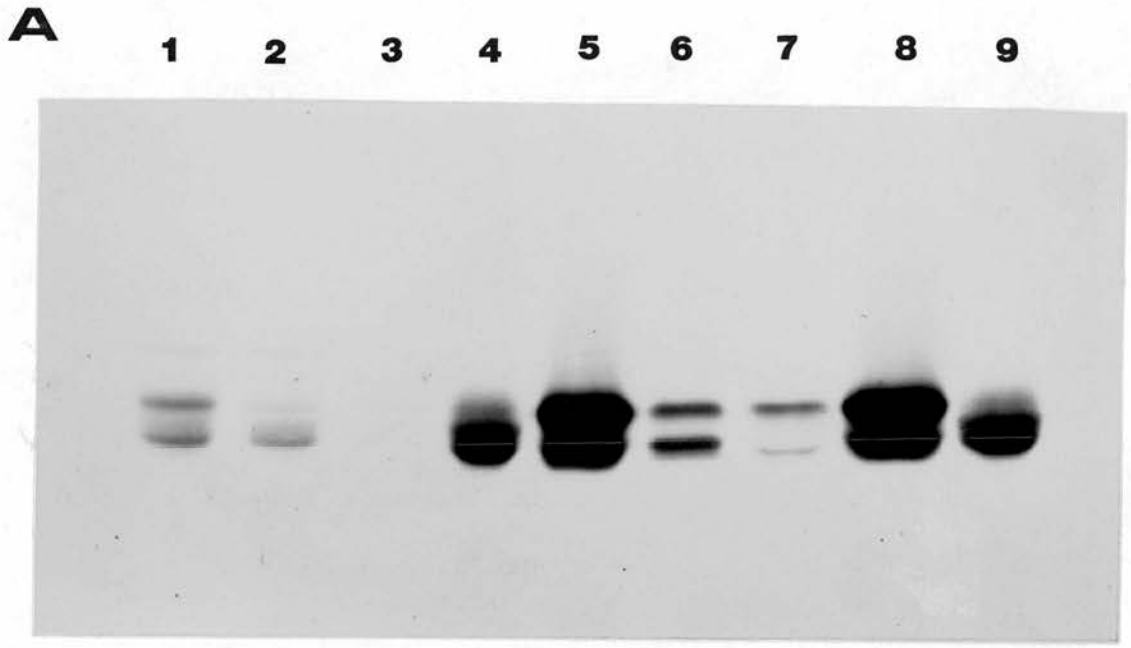
Figure 3.02.I

(ii) Immunoblotting of Bovine Adrenal Cortex Cytosol Preparations at Different Stages of Purification.

Antibodies raised against the polypeptides collected from peaks 1 and 2 described in fig. 3.02.B were used to blot against bovine adrenal cortex cytosols obtained at different stages during affinity chromatography (blots **A** and **B**, respectively). Also included in these immunoblots were various fractions purified from bovine adrenal cortex cytosol on S-hexylglutathione-Sepharose 6B, along with the GST isoenzyme pool purified on glutathione-Sepharose 6B. The initial gel loadings consisted of the following: lane 1, unfractionated bovine adrenal cortex cytosol; lane 2, flow-through fraction from the S-hexylglutathione-Sepharose 6B column; lane 3, flow-through fraction from the glutathione-Sepharose 6B column; lanes 4 and 9, GST isoenzyme pool purified on glutathione-Sepharose 6B; lanes 5 and 8, alpha-class GST pool purified on S-hexylglutathione-Sepharose 6B (see fraction 13, fig. 3.01.A); lane 6, pi-class GST purified on S-hexylglutathione-Sepharose 6B (fraction 23, fig. 3.01.A); lane 7, mu-class GST purified on S-hexylglutathione-Sepharose 6B (fraction 34, fig. 3.01.A)

Figure **3.02.I**

(ii)



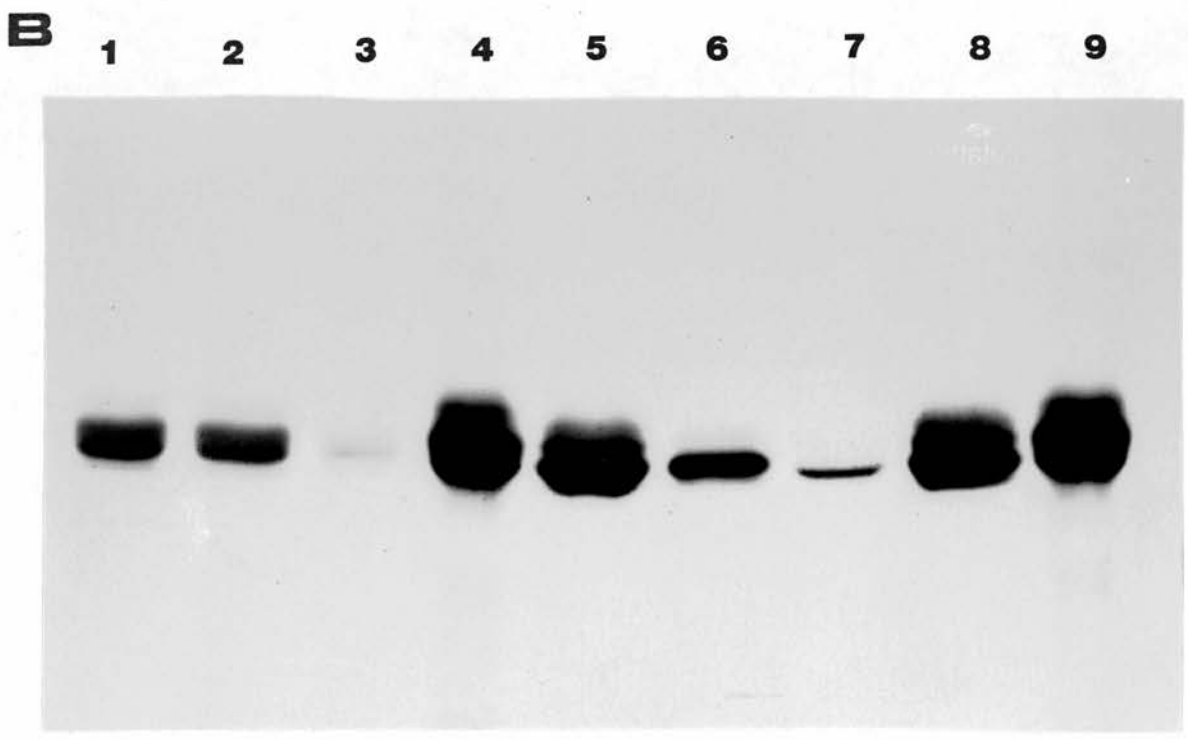


Table 3.02(B)

GST Activities of Bovine Adrenal Cortex Cytosols at Different Stages of Affinity Chromatography, as well as Activities of the Glutathione–Sepharose 6B Purified Pool, Using Different GST Substrates.

	GST Substrate (μmol/min/mg protein)			
	CDNB	CuOOH	ADD	OH–NON
Pre–column cytosol	0.24	0.23	0.015	0.065
S–hexG–Ag flow–through	0.09	0.26	0.014	0.061
GSH–Ag flow–through	0.01	0.13	0.001	N.S.
GST pool purified on GSH–Ag	9.59	19.2	1.67	5.09

Abbreviations:

S–hexG–Ag, S–hexylglutathione–Sepharose 6B; GSH–Ag, glutathione–Sepharose 6B; CDNB, 1–chloro–2,4–dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5androstene–3,17–dione; OH–NON, 4–hydroxynon–2–enal; N.S., not significant.

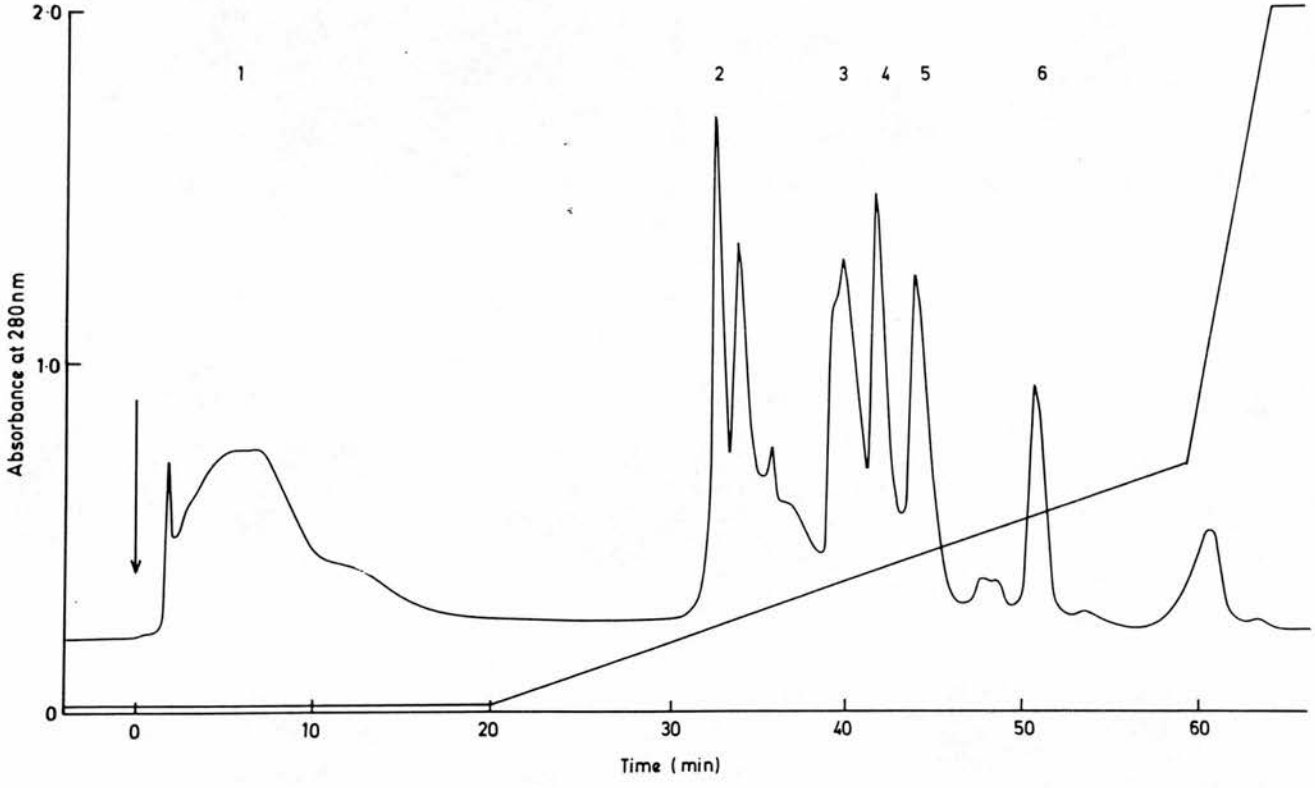
the anti(Ya_3) antiserum (blot B) revealed a very similar overall pattern of crossreactivity to blot A. However, in this blot, the pre-column cytosol and S-hexG-Ag column flow-through showed almost the same degree of cross-reactivity, which is almost absent in the flow-through fraction from the GSH-Ag column.

(iii) Anion-Exchange Chromatography of the Alpha-class GST Pool Purified on Glutathione-Sepharose 6B (GSH-Ag)

Several chromatographic matrices were used in attempts to further resolve the GST pool purified on GSH-Ag; the column matrices investigated included CM-cellulose, hydroxyappatite, cation-exchange chromatography and the μBondapak C_{18} reverse-phase h.p.l.c. column. However, the method which gave the best resolution was anion-exchange chromatography using the mono-Q anion-exchange column produced by Pharmacia (see "Methods"). Using this column at pH 8.5, the GSH-Ag purified pool was resolved into at least six different peaks (Fig. 3.02.J). SDS/PAGE analysis of selected fractions collected throughout each chromatographic run revealed that they contained a complex mixture of GST subunits (Fig. 3.02.K, gel A). In order to help determine the number of distinct subunits present in the fractions from the mono-Q column and to elucidate the total number of alpha-class subunits in bovine adrenal cortex, the apparently "pure" subunit fractions from anion-exchange chromatography were analysed by SDS/PAGE along with the alpha-class GST pools from S-hexG-Ag and GSH-Ag affinity chromatography (Fig. 3.02.K, gel B).

As a further aid to interpretting the results of anion-exchange chromatography, each fraction was assayed for GST activity using different model alpha-class GST substrates (Fig. 3.02.L, profiles (i) - (iv)). The flow-through fractions (peak 1, Fig. 3.02.L) showed activity with all substrates, whereas those fractions making up peaks 2-6 (as described in Fig. 3.02.L) varied considerably in their activities with the different substrates. This presents a complex situation, especially in the context of the number of distinct alpha-class subunits present; the significance of these findings will be discussed more fully in the next chapter.

Figure **3.02.J**



Anion-Exchange Chromatography of Bovine Adrenal Cortex GST Purified on Glutathione-Sepharose 6B.

Aliquots of the GST isoenzyme pool purified on glutathione-Sepharose 6B were applied to the Pharmacia FPLC system's mono-Q anion-exchange column which had been equilibrated with 0.25 mM Tris buffer (pH 8.5) containing 2 mM 2-mercaptoethanol. The limit buffer contained 0.5 mM NaCl, and the eluate was monitored at 280 nm. A flow-rate of 0.75 ml min was used, and 0.75 ml fractions were collected throughout each chromatographic run. Following application of the sample to the column (as indicated by the arrow) and a subsequent 20 min loading time, the bound isoenzymes were resolved on a 0-35% salt gradient formed over 40 min. Six main peaks were obtained during each run as indicated.

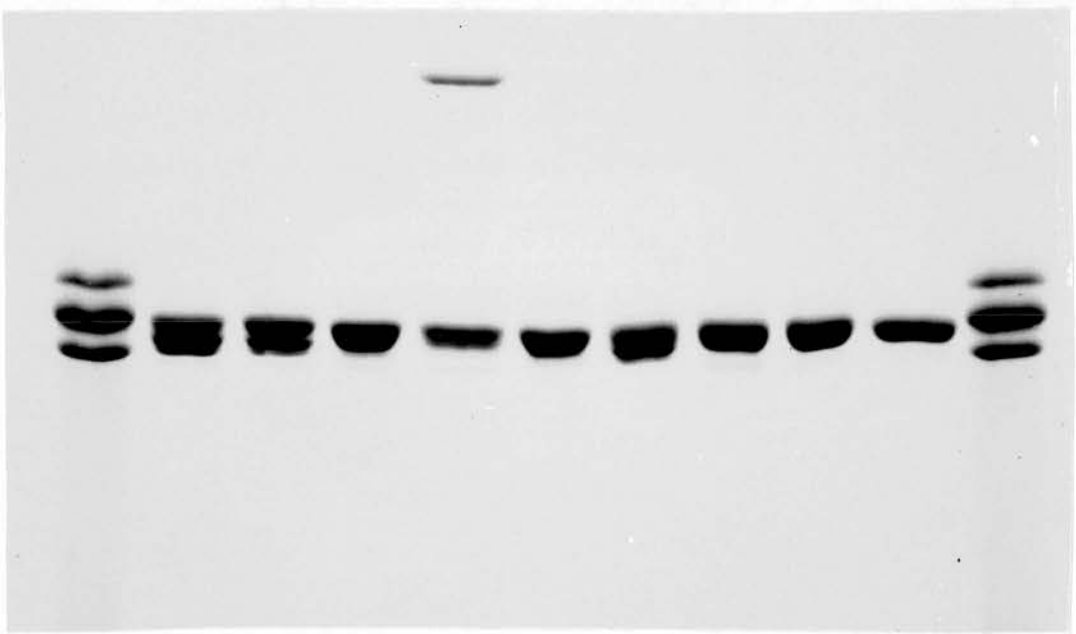
Figure 3.02.K

Electrophoretic Analysis of Fractions Collected During Anion-Exchange Chromatography of the GST Pool Purified on Glutathione-Sepharose 6B.

Various fractions collected during anion-exchange chromatography of the GST isoenzyme pool purified on glutathione-Sepharose 6B (see Fig. 3.02.J) were selected for analysis by SDS/PAGE. Gel (A) contained an analysis of individual fractions collected during each chromatographic run, consisting of the following loadings (see fig 3.02.L for corresponding positions in the elution profile): lanes designated "M" contained a rat liver isoenzyme mixture comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, fraction 7 from anion-exchange chromatography; lane 2, fraction 34; lane 3, fraction 35; lane 4, fraction 38; lane 5, fraction 40; lane 6, fraction 41; lane 7, fraction 43; lane 8, fraction 45; lane 9, fraction 51.
Gel (B) compared the electrophoretic mobilies of proteins in certain fractions collected during anion-exchange chromatography along with various GST isoenzyme markers from different stages of the purification process: lane "M" contained a rat liver GST isoenzyme mixture comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lanes 1 and 9, alpha-class GST purified on S-hexylglutathione-Sepharose 6B (see fig. 3.01.A); lanes 2 and 8, GST isoenzyme pool purified on glutathione-Sepharose 6B; lane 3, fraction 35 of anion-exchange chromatography; lane 4, fraction 40; lane 5, fraction 43; lane 6, fraction 45; lane 7, fraction 51.

Figure **3.02.K**

A



B

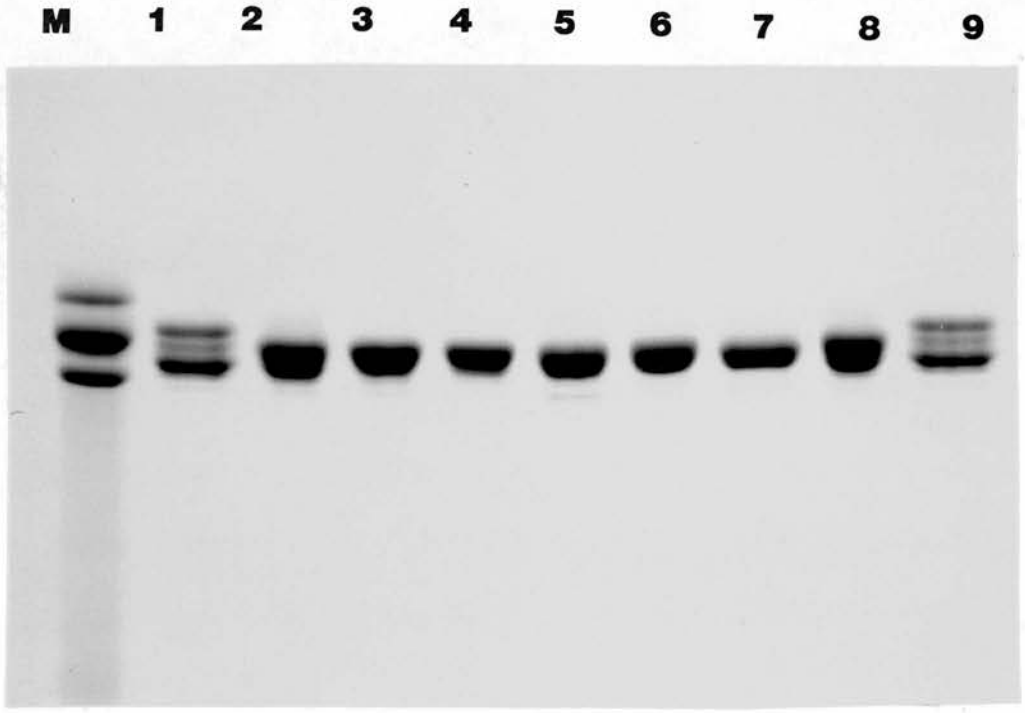


Figure 3.02.L (i-iv)

GST Activities of Fractions Collected During Anion-Exchange Chromatography of the Glutathione-Sepharose 6B Affinity-Purified Pool from Bovine Adrenal Cortex Using Different GST Substrates.

The fractions collected during anion-exchange chromatography of the GST isoenzyme pool purified on glutathione-Sepharose 6B were assayed for GST activity using a number of model substrates specific for alpha-class GST. The values obtained were used to plot elution profiles alongside values of protein concentration for each fraction: (i), with 1-chloro-2,4-dinitrobenzene (CDNB); (ii), with cumene hydroperoxide (CuOOH); (iii), with Δ^5androstene-3,17-dione (ADD) and (iv), with 4-hydroxynon-2-enal.

Figure 3.02.L(i)

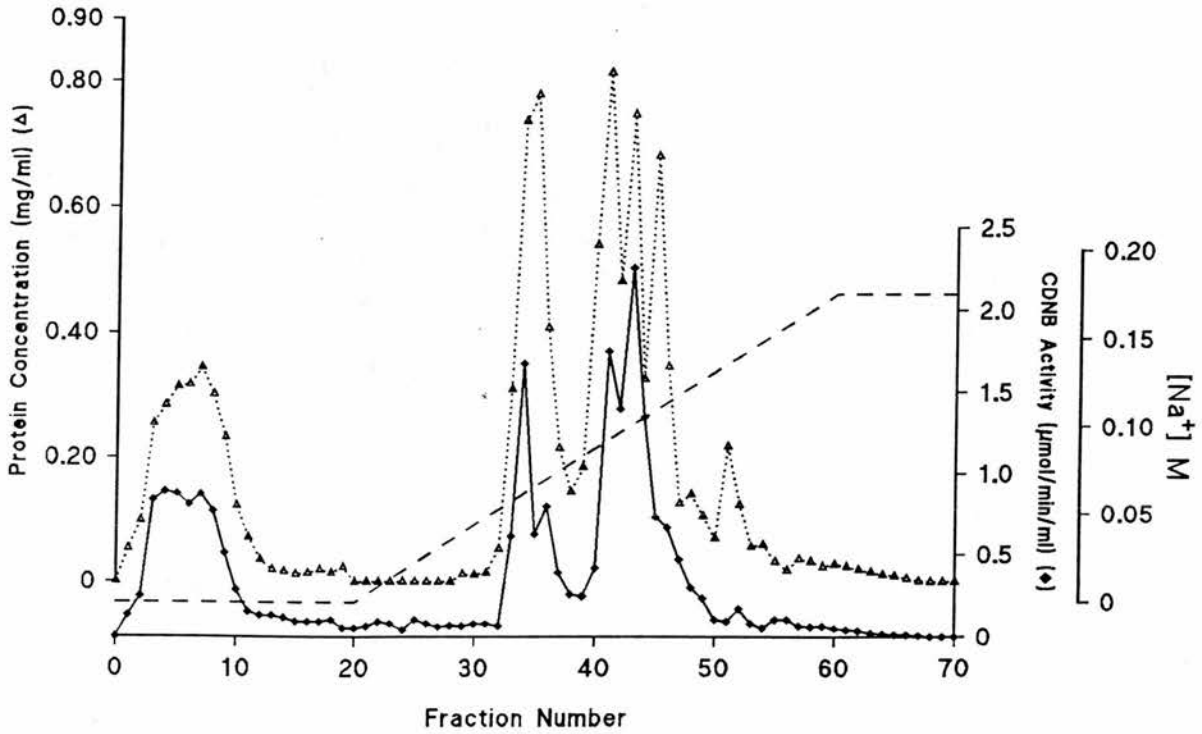


Figure 3.02.L(ii)

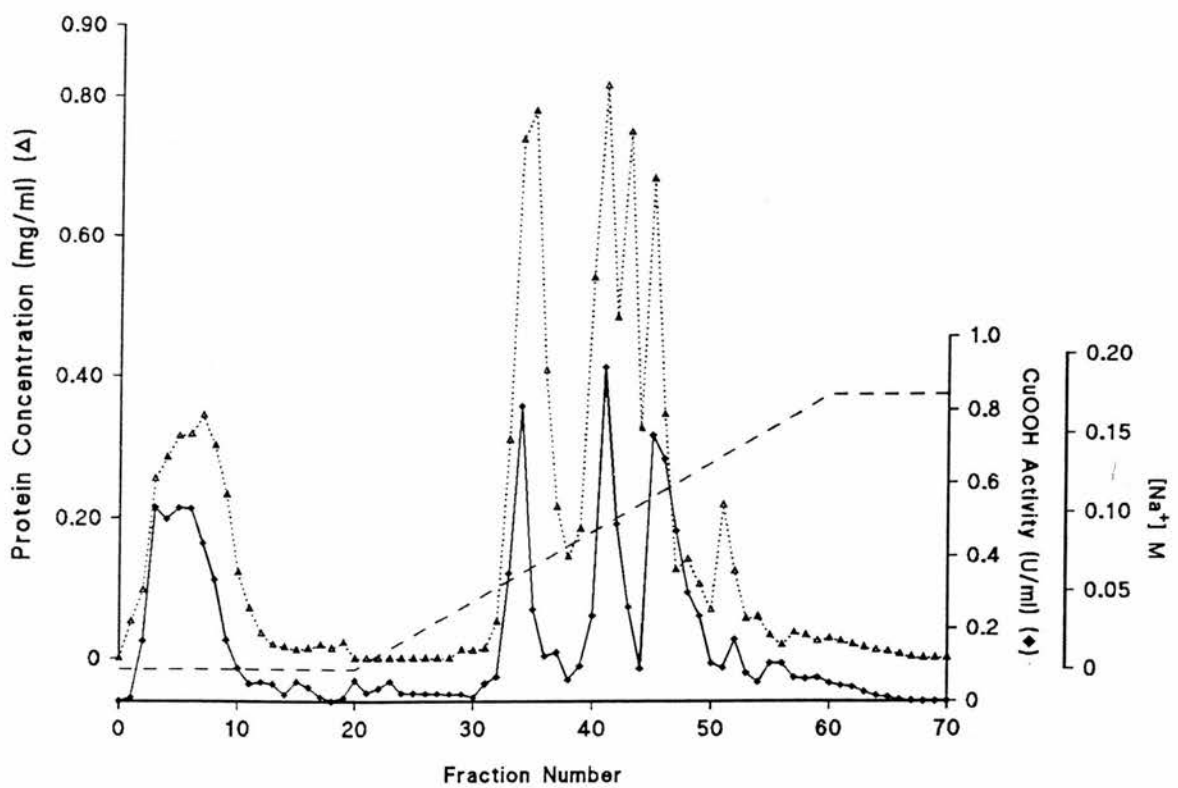


Figure 3.02.L(iii)

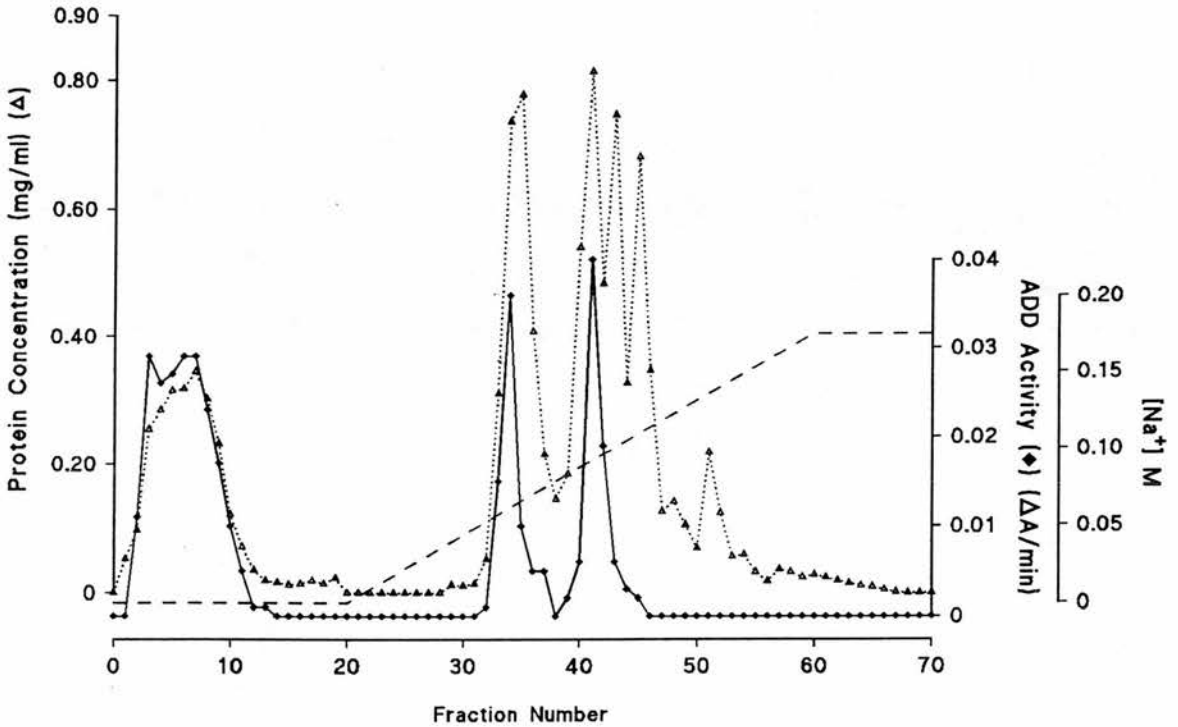
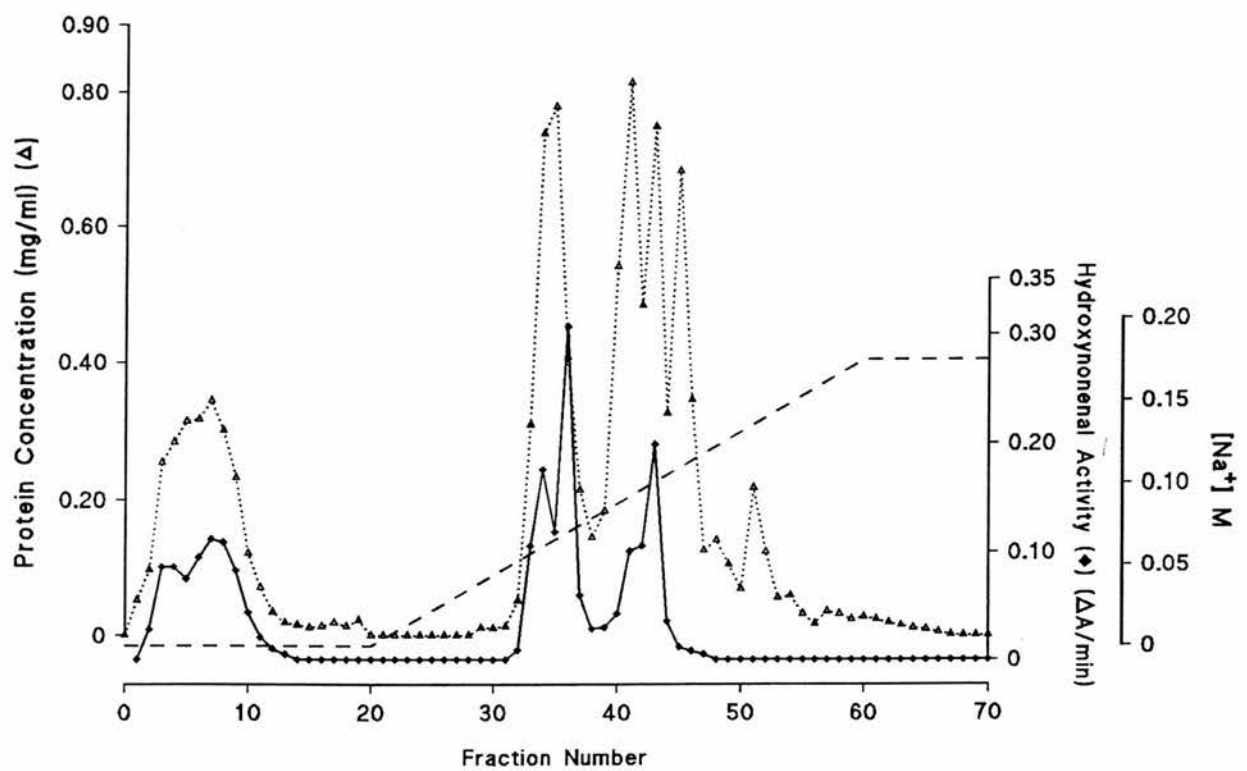


Figure 3.02.L(iv)



3.03 Expression of Alpha-class GST in Non-Adrenal Bovine Tissues

(i) SDS/PAGE Analysis

In view of the requirement for GSH-Ag to purify all the adrenocortical alpha-class GST, it was decided to re-examine the cytosols from a number of different bovine organs by SDS/PAGE (Fig. 3.03.A). In addition to treating the adrenal cortex and medulla separately in these experiments, the different zones of the cortex (i.e. zona fasciculata/reticularis and zona glomerulosa) were also analysed separately. The resulting gel showed the expression of an abundant protein(s) in the corresponding GST region in cytosols prepared from whole adrenal cortex tissue (lane 1), the zona fasciculata/reticularis of the adrenal cortex (lane 2), the zona glomerulosa of the adrenal cortex (lane 3), liver (lane 5) and, to a lesser extent, testes and adrenal medulla (lanes 4 and 7 respectively). Interesting differences were observed in the expression of GST between the different adrenal cortex zones: the zona fasciculata appeared to show slightly elevated levels of these GST isoenzymes compared to the whole adrenal cortex tissue cytosol; also, the slower-migrating band(s) appeared to be expressed to a lesser extent in the zona glomerulosa when compared to cytosol from both the zona fasciculata and whole adrenal cortex.

(ii) Immunoblotting

Immunoblotting was used with the same cytosolic fractions in an attempt to quantify the relative amount of alpha-class GSTs expressed in the different bovine tissues. In these blots, antisera which had been raised separately against the Ya_1 and Ya_3 subunits described earlier (Fig. 3.02.B) were used in blots A and B respectively (Fig. 3.03.B), as well as antiserum which had been raised against the alpha-class GST pool purified on GSH-Ag (blot C, Fig. 3.03.B). Anti(Ya_1) antiserum, showed cross-reactivity with several bands in the GST region of a number of different bovine tissues (blot A). This antibody appeared to cross-react with both subunits purified from S-hexG-Ag on reverse-phase h.p.l.c. and , as predicted from this result, marked cross-reactivity with two bands was observed in all lanes containing cytosols derived from the adrenal cortex (lanes 1, 2 and 3). Interestingly, this antibody cross-reacted with a third band located in-between the other two. This was especially evident in cytosols prepared from whole adrenal cortex and zona fasciculata. This protein is probably equivalent to the Mr 26 500 polypeptide purified on

Figure 3.03.A

Electrophoretic Analysis of Cytosolic Fractions Prepared from Different Bovine Organs.

The cytosolic fraction from a range of different bovine organs was prepared (see "methods") and analysed by SDS/PAGE. The gel loadings were as follows: lane "M" contained a rat liver GST isoenzyme mixture comprising Yc (Mr 27500), Yb (Mr 26300) and Ya (Mr 25500) subunits; lane 1, bovine adrenal cortex tissue cytosol; lane 2, cytosol prepared from the zona fasciculata/reticularis of bovine adrenal cortex; lane 3, cytosol prepared from the zona glomerulosa of bovine adrenal cortex; lane 4, cytosol prepared from bovine testes; lane 5, cytosol prepared from bovine liver; lane 6, cytosol prepared from bovine lung; lane 7, cytosol prepared from bovine adrenal medulla; lane 8, cytosol prepared from bovine spleen; lane 9, cytosol prepared from bovine kidney; lane 10, cytosol prepared from bovine heart.

Figure **3.03.A**

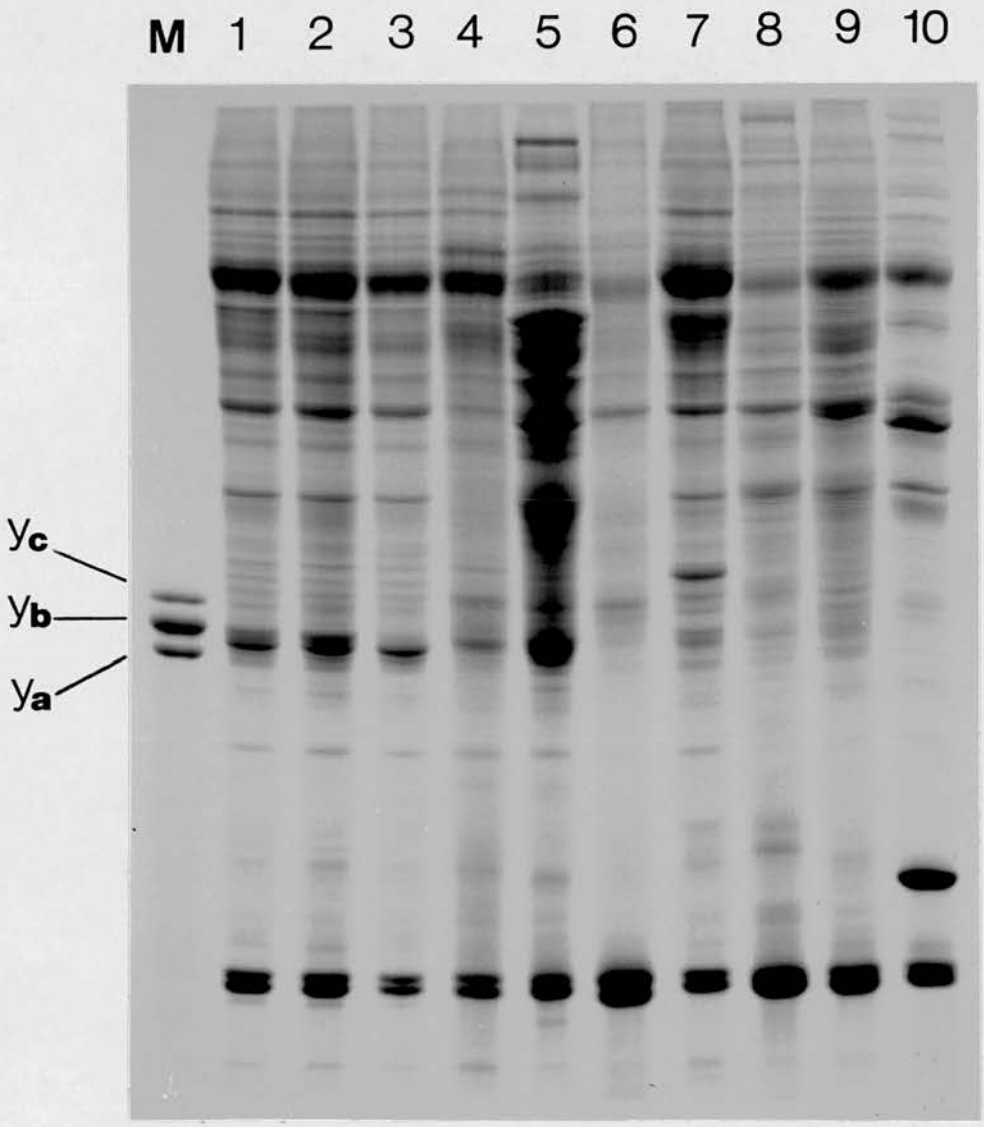
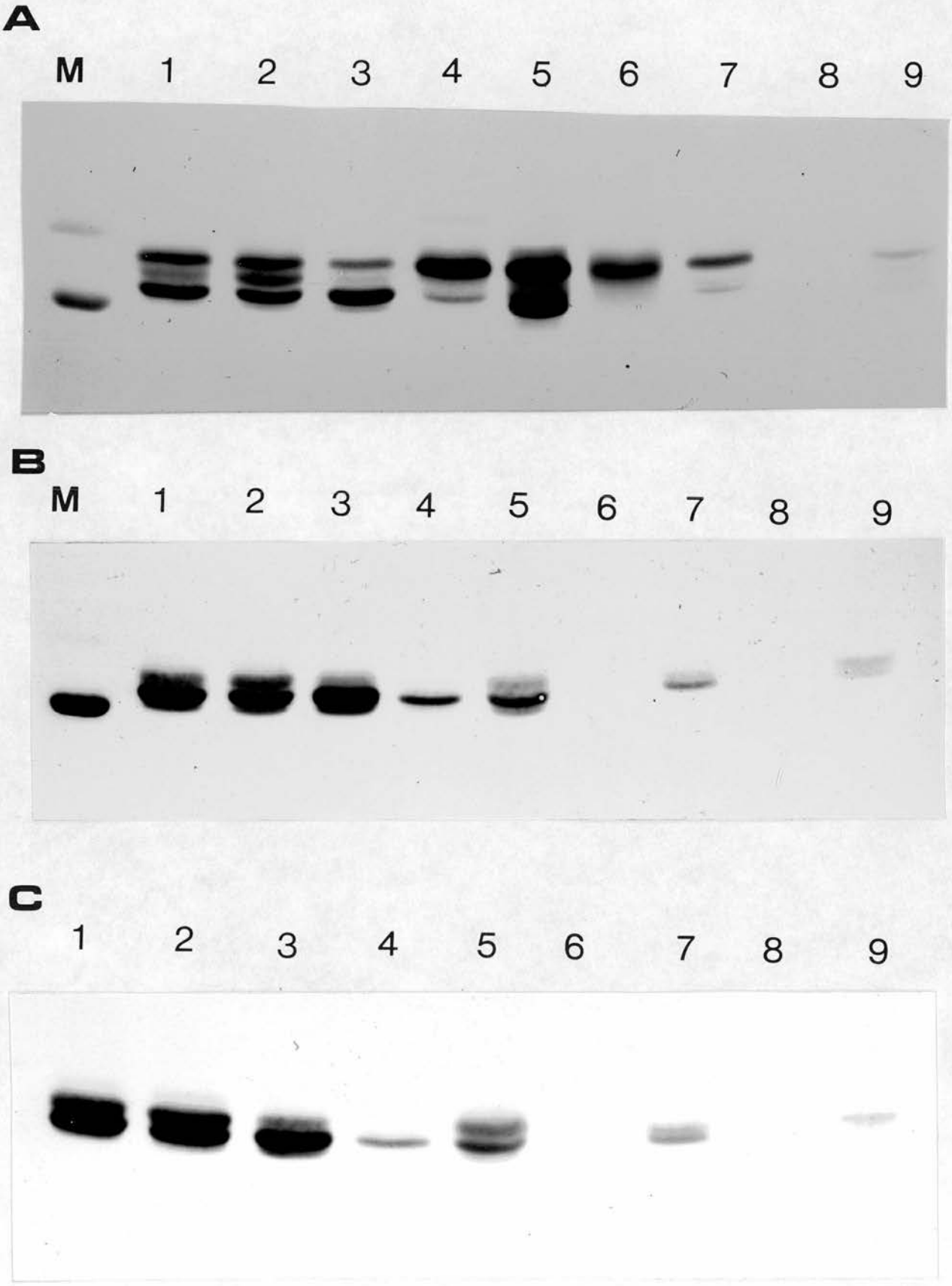


Figure 3.03.B

Immunoblotting of Cytosolic Fractions Prepared from Different Bovine Organs.

Antibodies raised against purified alpha-class GSTs were used to blot against cytosols prepared from a range of bovine organs. Blot **A** used the antibodies raised against one of the GST subunits purified by reverse-phase hplc from a partially purified alpha-class GST pool (peak 1, fig. 3.02.B); blot **B** used antibodies raised against the other of the GST subunits purified by reverse-phase hplc from a partially-purified GST pool (peak 2, fig. 3.02.B); blot **C** used antibodies raised against the GST isoenzyme pool purified on glutathione-Sepharose 6B (see fig. 3.02.I). The loadings for blots **A** and **B** were as follows: lane "M" contained a rat liver GST isoenzyme mixture described in fig. 3.03.A; lane 1, cytosol prepared from bovine adrenal cortex; lane 2, cytosol prepared from the zona fasciculata/reticularis of bovine adrenal cortex; lane 3, cytosol prepared from the zona glomerulosa of bovine adrenal cortex; lane 4, bovine testes cytosol; lane 5, bovine liver cytosol; lane 6, bovine lung cytosol; lane 7, bovine adrenal medulla cytosol; lane 8, bovine spleen cytosol; lane 9, bovine kidney cytosol. Blot **C** had the same loadings except for the exclusion of rat liver GST markers.

Figure **3.03.B**



GSH-Ag (Fig. 3.02.I). The liver cytosol also showed marked cross-reactivity, with at least two bands identified using this antibody (lane 5, blot A), and both testes and lung cytosols (lanes 4 and 6 respectively) showed relatively marked cross-reactivity with the appearance of the slower-migrating band on the Western blot. Both spleen (lane 8) and heart (result not shown) cytosols failed to show cross-reactivity with the anti(Ya_1) antiserum although, interestingly, the rat liver GST Ya subunit showed much higher cross-reactivity than the rat Yc subunit (lane M).

Blot B, involving the anti (Ya_3) antiserum, showed a similar overall pattern of cross-reactivity: all cytosols derived from the adrenal cortex had a high level of cross-reactivity with the appearance of the same two bands, although the slower band in the zona glomerulosa cytosol was much less evident (lane 3, blot B). The liver cytosol showed less cross-reactivity with this antibody than the adrenal cortex, as was the case for the testes cytosol in which only one band appeared to cross-react. Both the adrenal medulla and kidney cytosols showed only slight cross-reactivity, whereas the lung, spleen and heart cytosols possessed no cross-reactivity. The rat liver Ya subunit showed marked cross-reactivity with this antibody, although the Yc subunit did not cross-react at all. Blot C, carried out with the antiserum raised against the alpha-class GST pool purified on GSH-Ag, resulted in an overall pattern which was almost identical to blot B, although this antiserum did not cross-react with any of the rat liver GST subunits.

(iii) Purification of Alpha-class GSTs from Non-Adrenal Bovine Tissues Using Glutathione-Sepharose 6B (GSH-Ag)

The observations about tissue-specific expression of GST made during SDS/PAGE analysis and immunoblotting of cytosols from different bovine organs were given support by enzyme activity assays at different stages of affinity chromatography (table 3.03.A) of cytosols from the different tissues. For all tissues, except the adrenal cortex, liver and testes, most of the GST isoenzymes were purified on the S-hexG-Ag column alone, with little GST activity being found in the flow-through from this column. In the case of the liver, testes and adrenal cortex, significant GST activity was recovered in the S-hexG-Ag flow-through. Therefore, the GSTs remaining in the S-hexG-Ag flow-through fraction for both liver and testes were purified by application of this fraction to GSH-Ag, as described above for the adrenal cortex.

Table 3.03(A)

GST Activities Using CDNB for a Range of Tissue Cytosols at Different Stages of Affinity Chromatography.

(All specific activity values quoted are in units of μmol/min/mg protein)

Organ	Cytosol	S-hexG-Ag Flow-through	GSH-Ag Flow-through
Adrenal Cortex	0.24	0.09	0.01
Adrenal Medulla	0.10	0.02	N.S.
Liver	0.36	0.11	0.03
Testes	0.22	0.05	0.01
Lung	0.23	0.03	0.02
Spleen	0.14	0.02	0.02
Kidney	0.10	0.01	N.S.
Heart	0.12	0.01	0.01

(S-hexG-Ag denotes S-hexylglutathione-Sepharose 6B; GSH-Ag, glutathione-Sepharose 6B; N.S., not significant).

The elution profiles for both liver (Fig. 3.03.C) and testes (Fig. 3.03.E) on GSH-Ag were similar to the one obtained for the adrenal cortex (Fig. 3.02.H). Notably, however, the liver fractions contained much higher protein concentrations along with correspondingly higher GST activities using both CDNB and cumene hydroperoxide as substrates. As for the adrenal cortex, different stages of affinity chromatography for both liver and testes were analysed by SDS/PAGE. These stages included the GST pool purified on GSH-Ag along with the different isoenzymes purified on S-hexG-Ag. For the liver, abundant amounts of certain GST subunits remained in the flow-through fraction from the S-hexG-Ag column (lanes 1 and 2, Fig. 3.03.D). These subunits were removed following subsequent application of the flow-through to the GSH-Ag column (lane 3). Again, this was consistent with the GSH-Ag column having successfully bound these subunits. This was confirmed by demonstrating that the GSH-Ag purified pool (lane 4) consisted of two distinct polypeptides, apparently identical in mobility to the same subunits isolated from the adrenal cortex cytosol.

The situation was not quite so obvious for the testes (Fig. 3.03.F), since application of the cytosolic fraction to S-hexG-Ag appeared to remove the major protein(s) expressed in the GST region of the gel (see lanes 1 and 2, Fig. 3.03.F), with no apparent difference between the S-hexG-Ag and GSH-Ag column flow-through fractions (lanes 2 and 3). However, the GST polypeptides purified on GSH-Ag from bovine testes cytosol do show identical migration on SDS/PAGE to the two subunits from the equivalent enzyme pool in the adrenal cortex.

3.04 GST Isoenzymes in Human Adrenal Cortex

Having established that alpha-class GSTs predominate in bovine adrenal cortex, experiments were carried out to determine the GST complement of human adrenal cortex cytosol.

(i) Electrophoretic Analysis of Human Adrenal Cortex Cytosol

Cytosol prepared from human adrenal cortex tissue was analysed by SDS/PAGE on a gel which also contained cytosol from bovine adrenal cortex tissue, cytosols prepared separately from both zona glomerulosa and zona fasciculata of bovine adrenal cortex, and also the adrenal medulla (Fig. 3.04.A). Significantly, the human adrenal cortex expressed an abundant protein in

Figure 3.03.C (i/ii)

Single-Step Elution of Bovine Liver GST on Glutathione-Sepharose 6B.

The flow-through fraction from a column containing S-hexylglutathione-Sepharose 6B, following application of bovine liver cytosol, was reapplied to a column containing glutathione-Sepharose 6B. The GST isoenzymes bound to this column were eluted using a solution containing 40 mM GSH and collected into 6.5 ml fractions. Each fraction was assayed for both protein concentration and GST activity (using both CDNB and CuOOH as substrates).

Figure 3.03.C(i)

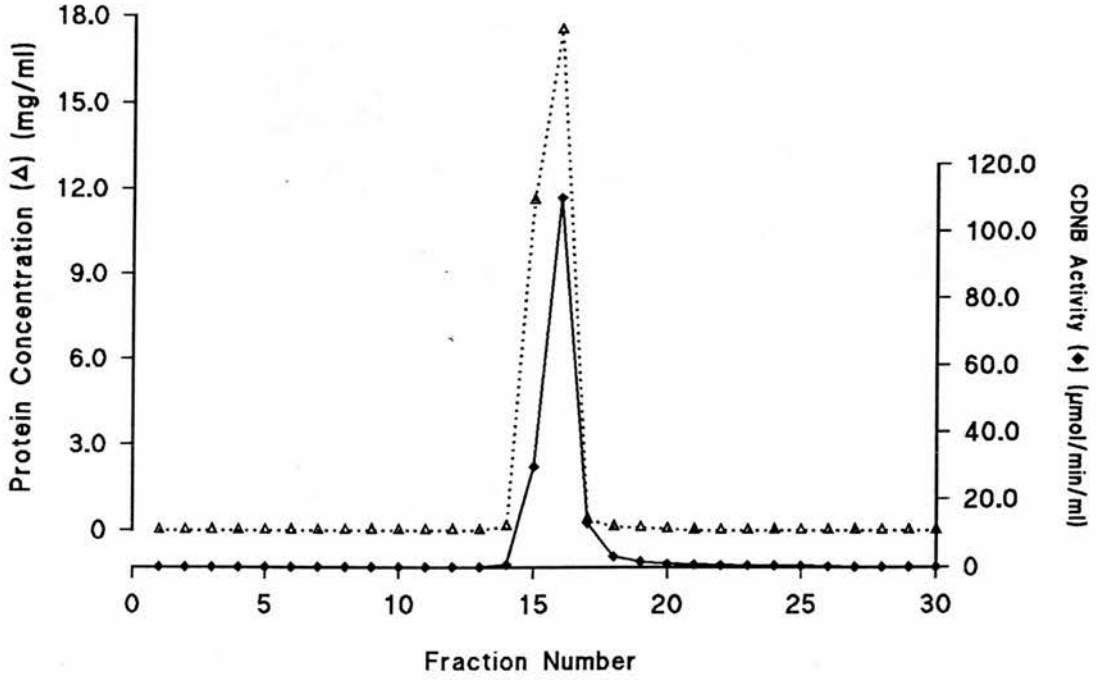


Figure 3.03.C(ii)

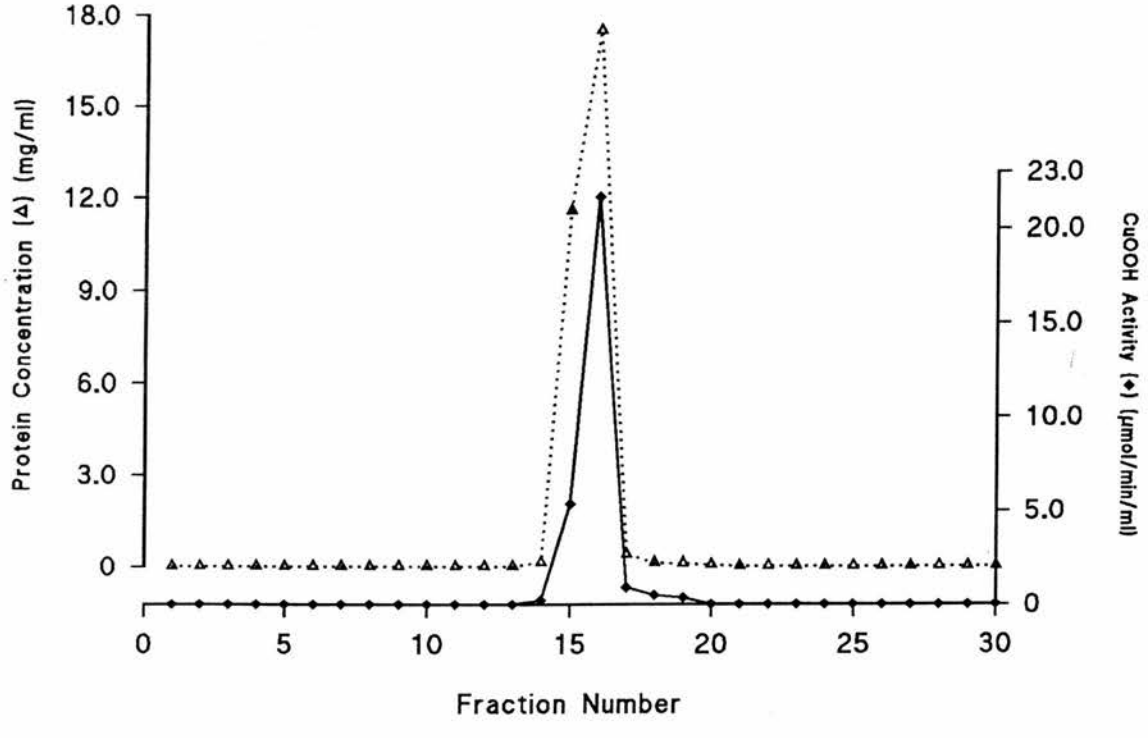


Figure 3.03.D

Electrophoretic Analysis of Bovine Liver Cytosols at Different Strages of Affinity Chromatography, Along with Various GST Isoenzymes purified at Each Stage.

Bovine liver cytosols (40 μg protein each) at different stages during affinity chromatography were analysed by SDS/PAGE. In addition, the GST isoenzymes purified at each affinity chromatography step were also included. The gel loadings were as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lane 1, unfractionated bovine liver cytosol; lane 2; flow-through fraction from the S-hexylglutathione-Sepharose 6B column; lane 3, flow-through fraction from the glutathione-Sepharose 6B column; lane 4, GST isoenzyme pool purified on glutathione-Sepharose 6B; lanes 5, 6 and 7, GST isoenzymes purified on S-hexylglutathione-Sepharose 6B (fig. 3.01.C, fractions 14, 33 and 46 respectively).

Figure **3.03.D**

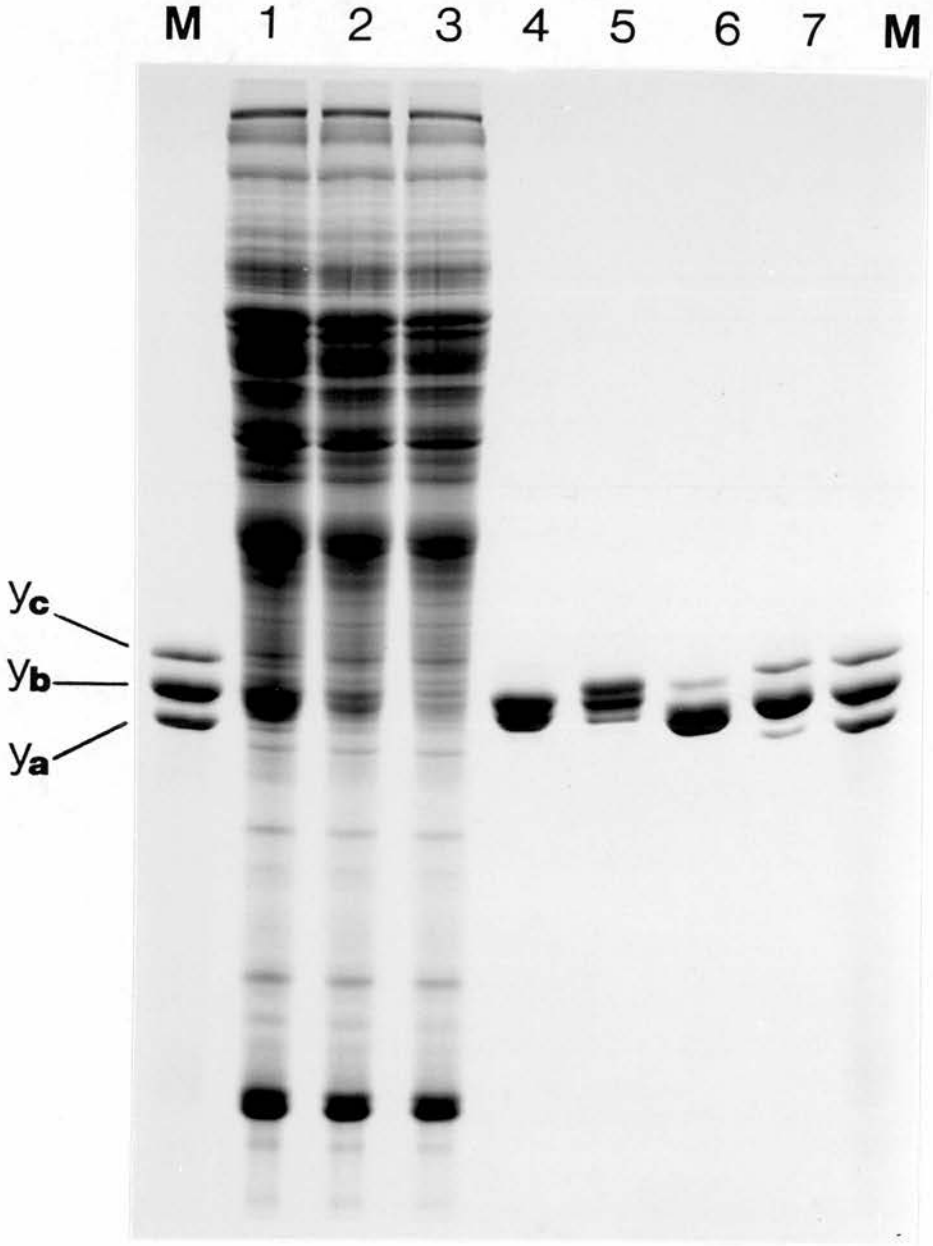


Figure 3.03.E (i/ii)

Single-Step Elution of Bovine Testes GST on Glutathione-Sepharose 6B.

The flow-through fraction from a column containing S-hexylglutathione-Sepharose 6B, following application of bovine testes cytosol, was reapplied to a column containing glutathione-Sepharose 6B. The GST isoenzymes bound to this column were eluted using a solution containing 40 mM GSH and collected into 6.5 ml fractions. Each fraction was assayed for both protein concentration and GST activity (using both CDNB and CuOOH as substrates).

Figure 3.03.E(i)

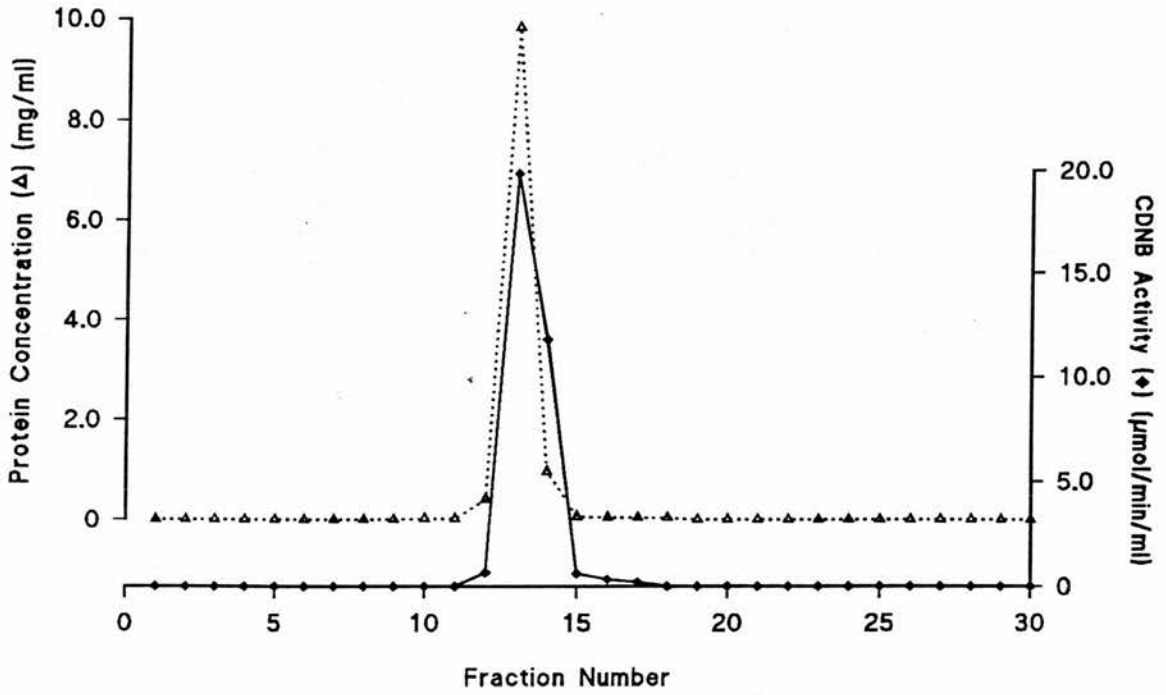


Figure 3.03.E(ii)

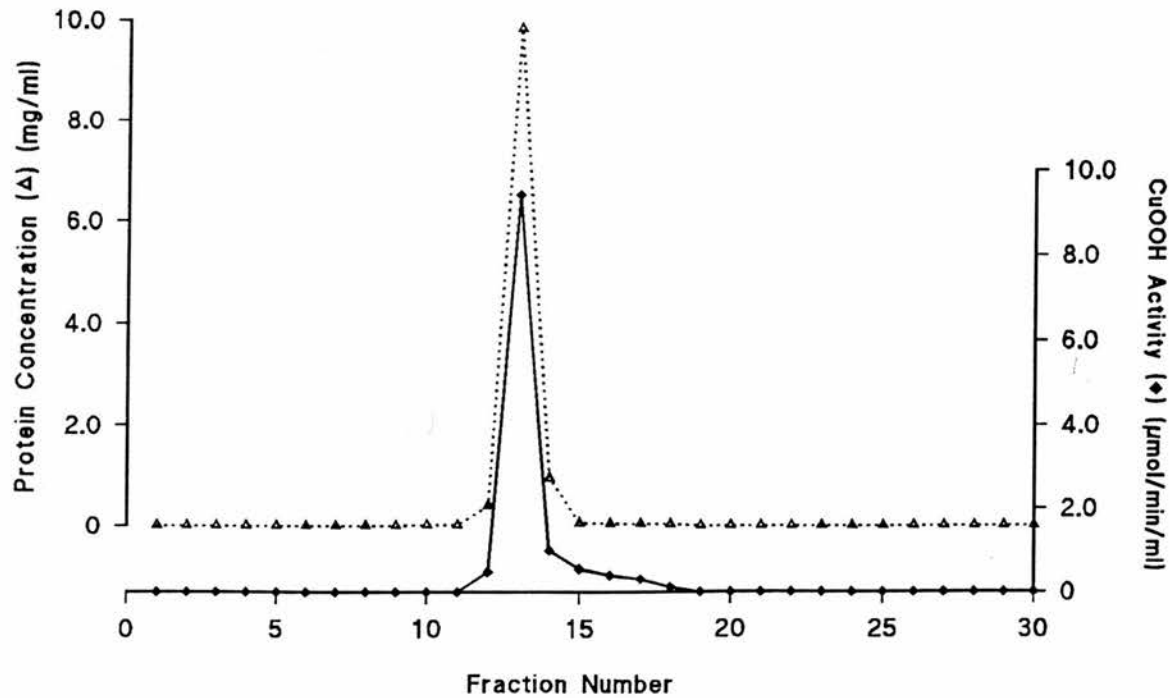
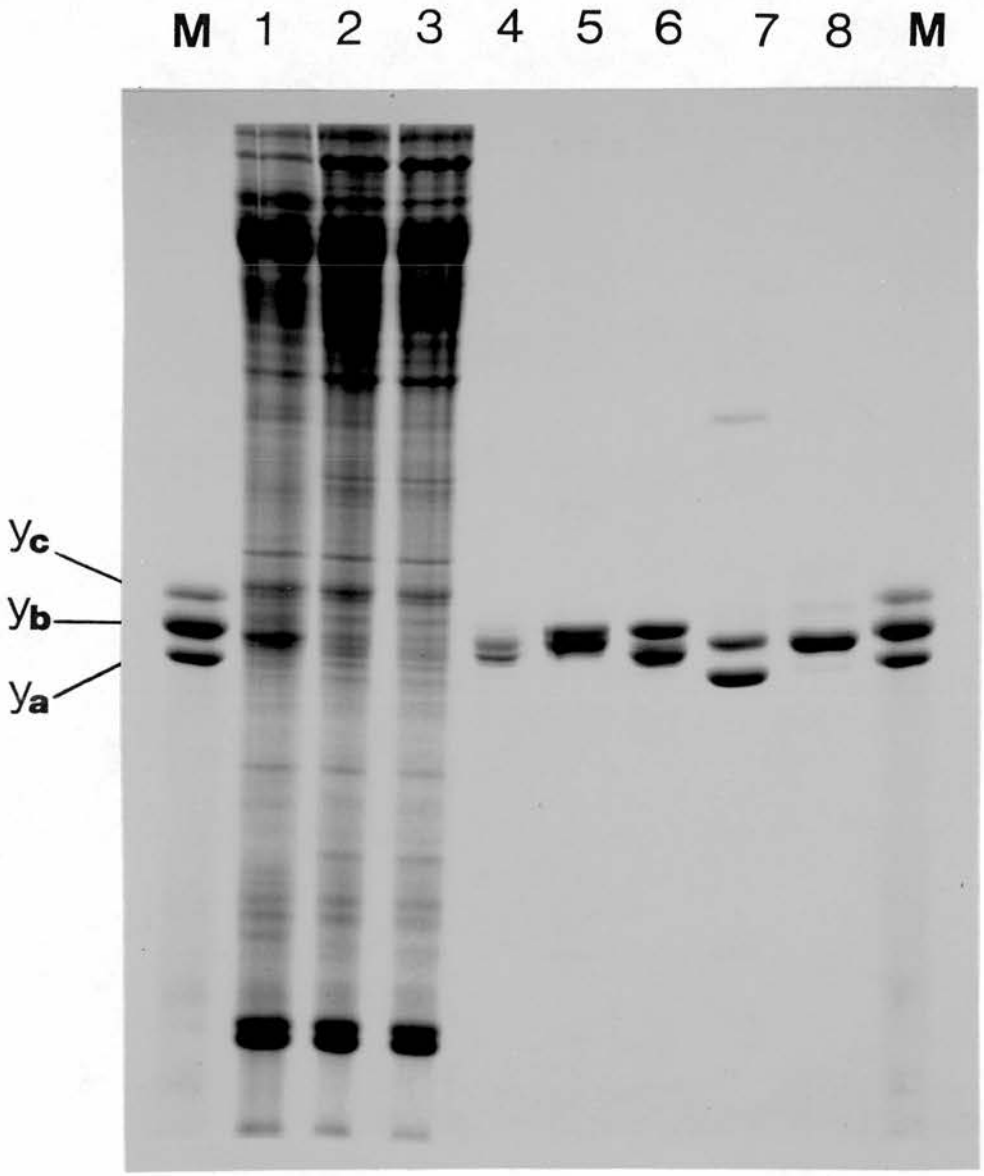


Figure 3.03.F

Electrophoretic Analysis of Bovine Testes Cytosols at Different Stages of Affinity Chromatography, Along with Various GST Isoenzymes Purified at Each Stage.

Bovine testes cytosols (50 μg protein each) at different stages during affinity chromatography were analysed by SDS/PAGE. In addition, the GSTs purified at each stage were also included. The gel loadings were as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lane 1, unfractionated bovine testes cytosol; lane 2, flow-through fraction from the S-hexylglutathione-Sepharose 6B column; lane 3, flow-through fraction from the glutathione-Sepharose 6B column; lane 4, GST isoenzyme pool purified on glutathione-Sepharose 6B; lanes 5, 6, 7, and 8, GST isoenzymes purified on S-hexylglutathione-Sepharose 6B (see fig. 3.03.E, fractions 13, 22, 25 and 52 respectively).

Figure **3.03. F**



the GST region of the gel (lanes 1, 2, 7 and 8) which further appeared to have a similar electrophoretic mobility to the faster-migrating alpha-class GST subunit previously described for bovine adrenal cortex.

In order to evaluate the significance of this finding, the human adrenal cortex cytosol was compared to human liver cytosol during SDS/PAGE (Fig. 3.04.B). Included in this gel were bovine adrenal cortex cytosol, rat liver GST markers and also purified human liver basic GST, the latter of which was intended to provide a source of enzyme which had been previously characterised and could thus serve as a protein standard. Cytosols from each of the three tissues showed evidence of abundant levels of proteins in the GST region (i.e. Mr 25000-27500). Bovine adrenal cortex cytosol (lane 3) showed the bands described previously, whilst both human liver and human adrenal cortex cytosol (lanes 1 and 2 respectively) also showed prominent staining of one band in this region. This latter human polypeptide band appeared to have a very similar electrophoretic mobility to the fast-migrating bovine alpha-class GST subunit described above. Furthermore, comparison with the human liver alpha-class GST B_1B_1 standard (lane 4) showed that the abundant electrophoretic band in both human liver and human adrenal cortex cytosols had very similar mobility, again consistent with this band being an alpha-class GST.

(ii) Affinity Chromatography of Human Adrenal Cortex GST

Human adrenal cortex cytosol was subjected to affinity chromatography using the same approach employed for the corresponding bovine cytosols: initial application of the cytosol to S-hexG-Ag, followed by re-application of the flow-through from this column to GSH-Ag. SDS/PAGE analysis of the cytosol at different stages of affinity chromatography (Fig. 3.04.C) revealed that the prominent band observed in the GST region using cytosol from this tissue was clearly removed following application to S-hexG-Ag. There was no obvious GST remaining in the flow-through from this column (compare lanes 1 and 2), in contrast to the findings for bovine adrenal cortex. As expected from this result, application of flow-through from the S-hexG-Ag column to GSH-Ag did not result in the further purification of GSTs (lane 3).

These observations were supported by measurement of GST activity at the different stages of purification using different substrates (Table 3.04.A). Almost all activity (with CDNB) was

Figure 3.04.A

Electrophoretic Analysis of the Cytosolic Fraction Prepared from Human Adrenal Cortex.

The cytosolic fraction from human adrenal cortex tissue was prepared (see "methods") and analysed by SDS/PAGE. Included in the gel for comparison were cytosols prepared from different layers of the adrenal gland, the gel loadings being as follows; lane "M" contained a rat liver GST isoenzyme mixture; lanes 1, 2, 7, and 8, human adrenal cortex cytosol; lane 3, cytosol prepared from the zona fasciculata/reticularis of bovine adrenal cortex; lane 4, cytosol prepared from the zona glomerulosa of bovine adrenal cortex; lane 5, cytosol prepared from bovine adrenal medulla; lane 6, cytosol prepared from whole bovine adrenal cortex.

Figure **3.04.A**

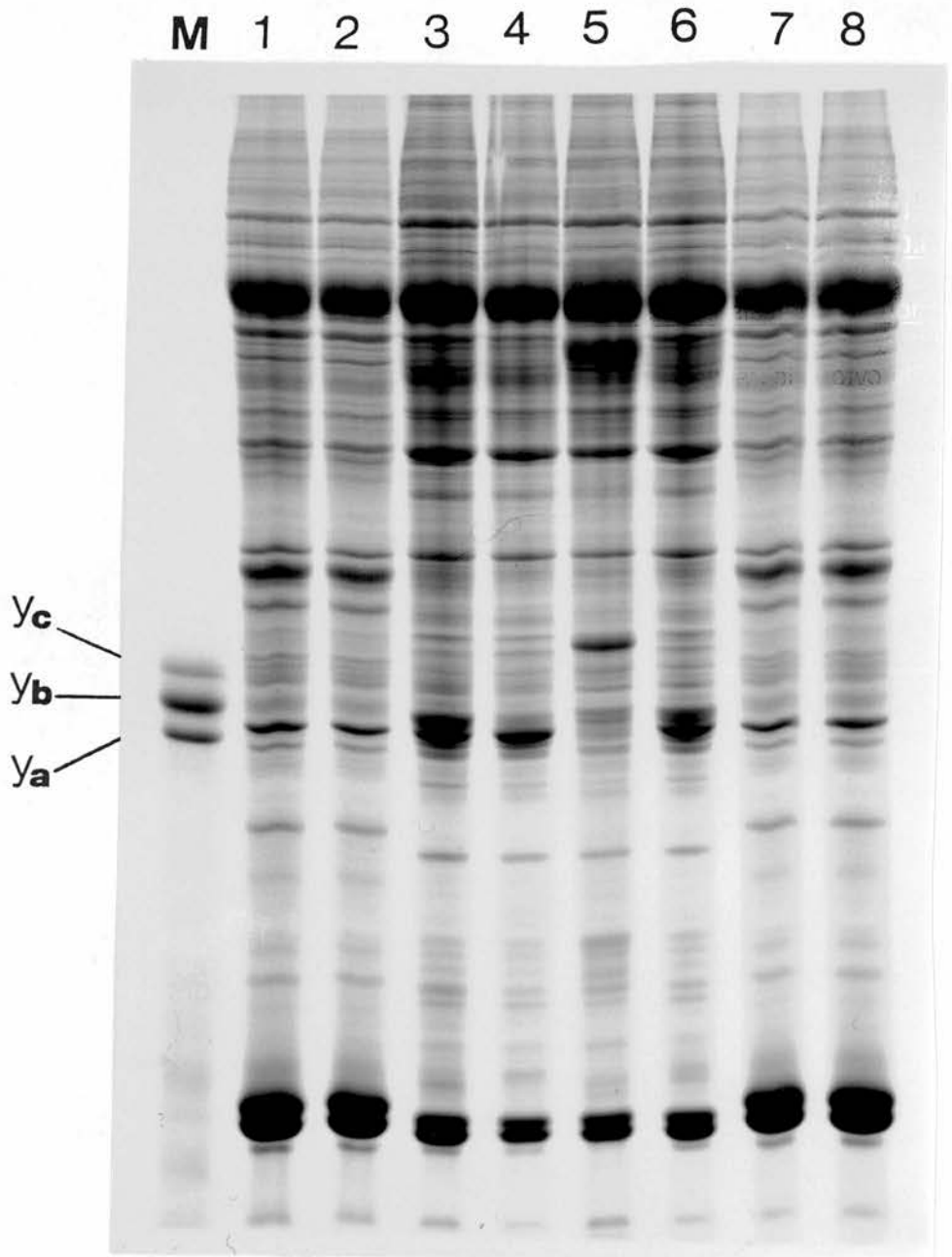
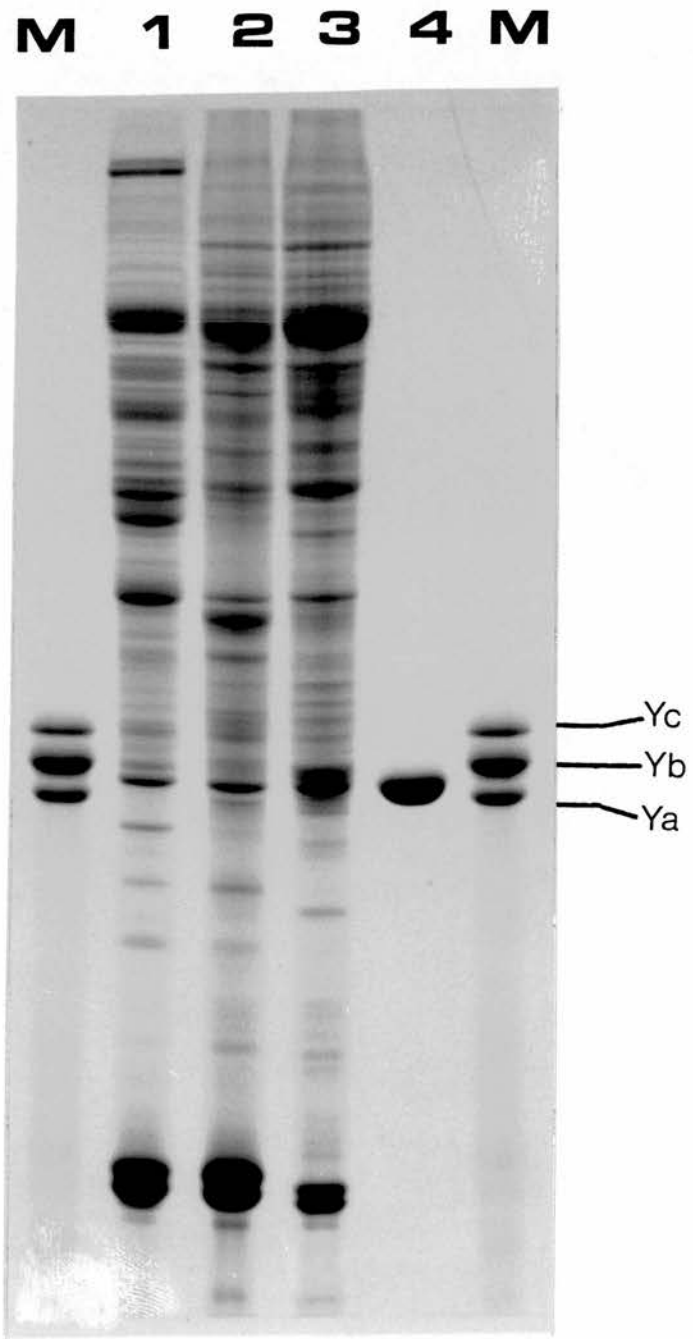


Figure 3.04.B

Electrophoretic Analysis of Human Liver, Human Adrenal Cortex and Bovine Adrenal Cortex Cytosol Preparations.

Cytosolic fractions from human liver, human adrenal cortex and bovine adrenal cortex tissues were analysed by SDS/PAGE. In addition to the rat liver GST marker, human liver GST B_1B_1 (Mr 25900) was included as a marker. The loadings were as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lane 1, human liver cytosol (50 μg protein); lane 2, human adrenal cortex cytosol (60 μg protein); lane 3, bovine adrenal cortex cytosol (60 μg protein); lane 4, purified human liver GST B_1B_1 marker.

Figure **3.04.B**



removed from the cytosol during the first stage of affinity chromatography on S-hex-Ag, with very little remaining in the flow-through from either affinity column. Similarly, using both cumene hydroperoxide and Δ^5-androstene-3,17-dione as substrate, activity was almost completely absent in the flow-through from the S-hexG-Ag column. Hence, unlike bovine adrenal cortex, all alpha-class GST activity appears to be completely retained on S-hexG-Ag using cytosol from human adrenal cortex. The peak CDNB activity fractions eluted from the S-hexG-Ag column were pooled (approximately 1.6% total cytosolic protein) for further analysis (results not shown). The purified pool on SDS/PAGE consisted almost entirely as one band with an apparent Mr of 25 900 (lane 4, Fig. 3.04.C), which is in agreement with the known electrophoretic mobilities of the GST B_1 and B_2 subunits from human liver cytosol **(Stockman *et al.*, 1985)**. The additional band found for the GSH-Ag purified pool from bovine adrenal cortex was not evident.

The purified pool was also found to be highly active with CDNB, in addition to the known alpha-class substrates, cumene hydroperoxide and Δ^5androstene-3,17-dione (Table 3.04.A). Further comparison of specific enzyme activities for the human adrenal cortex GST with those of human liver GST B_1B_1 revealed a marked similarity between the two enzymes (see Table 3.04.A for comparison).

The purified GST from human adrenal cortex cytosol was analysed by SDS/PAGE on a gel containing purified human GST markers from all three GST classes (Fig. 3.04.D). As expected from previous observations (SDS/PAGE analysis of human adrenal cortex cytosol, Figs. 3.04.B and 3.04.C; activity assays using different substrates specific for alpha-class GST, Table 3.04.A), the human adrenal cortex GST showed a very similar electrophoretic mobility to the human alpha-class standard (compare lanes 3, 4, 6 and 7 in Fig. 3.04.D). Interestingly, the GSH-Ag purified bovine adrenal cortex alpha-class pool also had a similar electrophoretic mobility to the human alpha-class standard.

(iii) Reverse-phase h.p.l.c. Analysis of Human Adrenal Cortex GST

Evidence that the purified human adrenal cortex GST comprised almost entirely of alpha-class GST was obtained by comparing the elution positions of this GST with purified human liver GST B_1B_2 during reverse-phase h.p.l.c. analysis (Fig. 3.04.E). Application of the human adrenal

Table 3.04(A)

GST Activities of Human Adrenal Cortex Cytosols at Different Stages of Affinity Chromatography, as well as Activities of the S-hexylglutathione-Sepharose 6B Purified Pool, Using Different GST substrates.

	GST Substrate (μmol/min/mg protein)		
	CDNB	CuOOH	ADD
Pre-column cytosol	0.64	0.34	0.075
S-hexG-Ag flow-through	0.06	0.08	N.S.
GSH-Ag flow-through	0.03	0.07	N.S.
GST pool purified on S-hexG-Ag	73	27	3.97
Human Liver GST B_1B_1	82*	31*	4.02

* Data for these substrates is taken from Stockman *et al* (1987)

Abbreviations:

S-hexG-Ag, S-hexylglutathione-Sepharose 6B; GSH-Ag, glutathione-Sepharose 6B; CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5androstene-3,17-dione; N.S., not significant.

Figure 3.04.C

Electrophoretic Analysis of Human Adrenal Cortex Cytosol Preparations at Different Stages of Affinity Chromatography.

Human adrenal cortex cytosols (60 μg protein) at different stages of affinity chromatography, along with the GST pool purified on S-hexylglutathione-Sepharose 6B, were analysed by SDS/PAGE. The gel was loaded as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lane 1, unfractionated human adrenal cortex cytosol; lane 2, flow-through fraction from the S-hexylglutathione-Sepharose 6B column; lane 3, flow-through from the glutathione-Sepharose 6B column; lane 4, purified enzyme from affinity-chromatography on S-hexylglutathione-Sepharose 6B.

Figure **3.04.c**

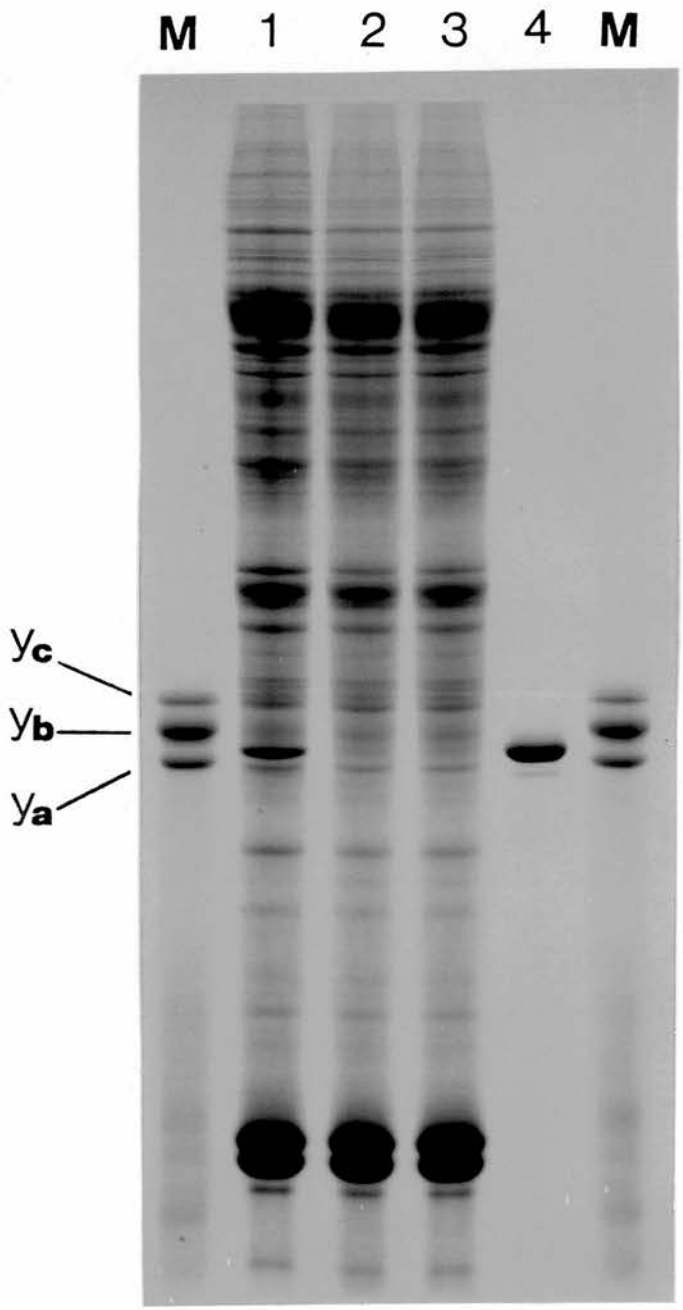
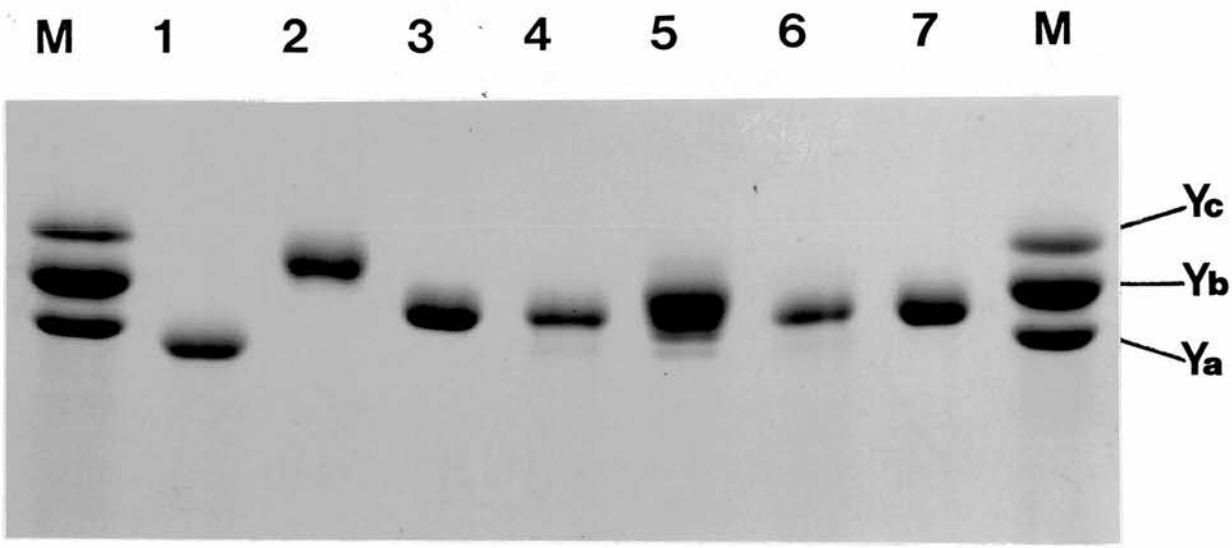


Figure **3.04.D**



Electrophoretic Analysis of the GST Isoenzyme Pool from Human Adrenal Cortex Purified on S-Hexylglutathione-Sepharose 6B.

The GST isoenzyme pool purified on S-hexylglutathione-Sepharose 6B was analysed by SDS/PAGE. Included in this gel for comparison were purified human GST subunit markers and the bovine adrenal cortex GST purified on glutathione-Sepharose 6B. The gel loadings were as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lane 1, human pi-class GST marker; lane 2, human mu-class GST marker; lanes 3 and 7, human alpha-class (B_1B_2) GST marker; lanes 4 and 6, GST purified from human adrenal cortex; lane 5, bovine adrenal cortex GST purified on glutathione-Sepharose 6B.

Figure 3.04.E (i-iii)

Analysis of Human Adrenal Cortex GST by Reverse-Phase hplc.

Reverse-phase hplc was employed to further characterise the GST isoenzyme pool purified from human adrenal cortex tissue cytosol. The GST expressed in this tissue was identified as being equivalent to human liver GST B_1B_1 by sequentially running purified human liver GST B_1B_2 (i), human adrenal cortex GST purified on S-hexylglutathione-Sepharose 6B (ii), and finally human adrenal cortex plus human liver GST B_1B_2 (iii). The column employed was a Waters μBondapak C_{18} column (10 μm particle size; 0.39 x 30 cm) which was developed at 1 ml/min using a linear 40-58% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 60 min. This was followed by a 58-70% acetonitrile gradient in aq. 0.1% trifluoroacetic acid formed over 5 min. The eluate was monitored continuously at 220 nm. The relative output of pump B is shown by the continuous line; pump A delivered 40% acetonitrile, pump B 70%. The arrows indicate the point of injection of the sample.

Figure 3.04.E

i

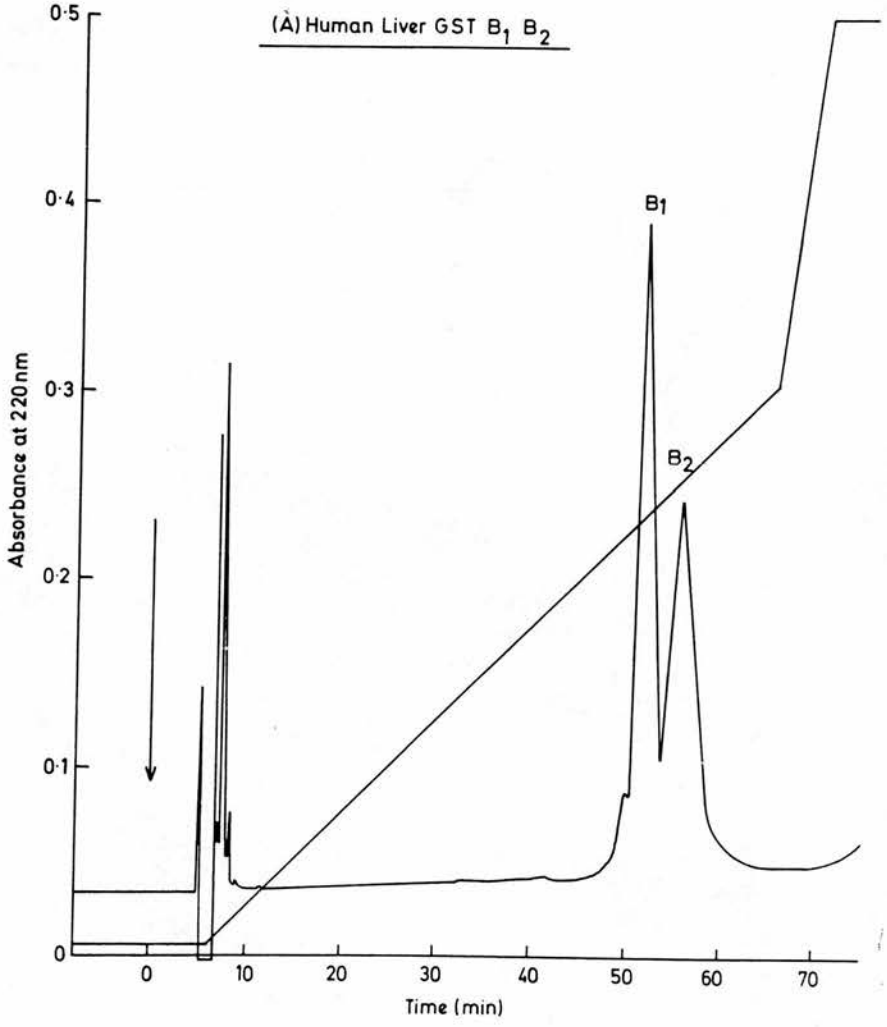


Figure **3.04.E**

ii

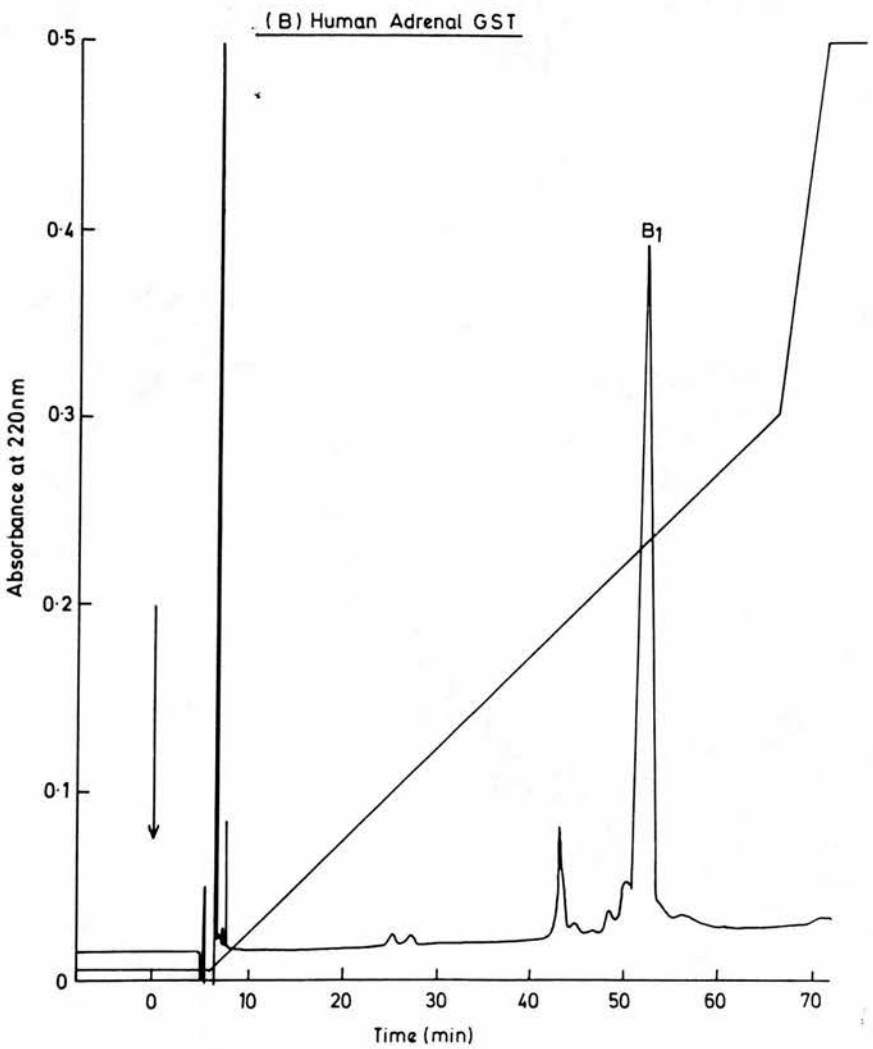
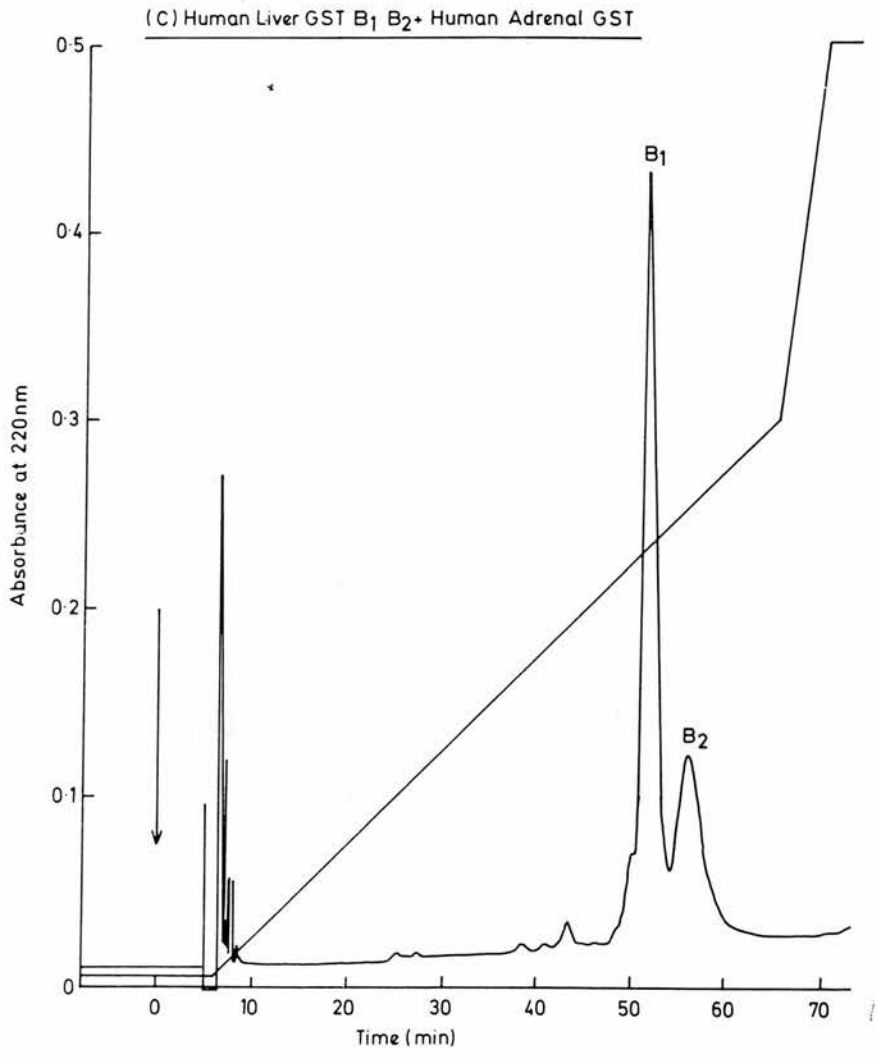


Figure **3.04.E**

iii



cortex GST pool, followed by subsequent gradient elution, led to the appearance of one main peak (Fig. 3.04.E(ii)). Previous application of human liver GST B_1B_2 established that the B_1 subunit co-eluted with this single GST peak (Fig. 3.04.E(i)). Simultaneous application of the human adrenal cortex GST pool with human liver B_1B_2 produced a marked elevation of the B_1 peak alone (Fig. 3.04.E(iii)). Hence, the GST subunit isolated from human adrenal cortex cytosol is probably identical to the B_1 subunit expressed in human liver.

3.05 GST Expression in Bovine Adrenocortical Cells in Primary Culture

The abundance of alpha-class GSTs in the adrenal cortex implies a major role for these enzymes in adrenocortical function. The final set of experiments were devised to investigate both the effects of prolonged primary culture of bovine adrenocortical cells and the effects of known steroidogenic agonists on expression of this GST subclass.

(i) Day-by-day Study

Initial experiments were carried out to determine whether GST expression changed during prolonged culture, since this would be an important factor in determining when to stimulate the cells with steroidogenic agonists. GST activities (with CDNB) of cytosols from adrenocortical cells on different days of primary culture gave values which were dependent upon the method used to harvest the cells (Fig. 3.05.A). The specific activities were considerably lower when a manual scraper was used to detach the monolayer. Using this method, GST activity was highest on day 1 of culture (i.e. freshly, collagenase-dispersed cells in suspension), fell on day 2, increased again by day 3, and then gradually decreased thereafter to day 7. When trypsin (0.01%) was used to harvest the adrenocortical cells, GST activity was relatively low on day 1, increased to a maximum on day 2, showed a slight drop on day 3, and then gradually decreased from day 4 onwards. Analysis of the surrounding medium into which the cells were harvested using both methods revealed a high value for GST activity in this medium when using the manual cell scraper (results not shown), suggesting that this method resulted in damage of the cells with a release of cytosolic contents. In the case of trypsin, little or no GST activity was detectable in the surrounding medium. Consequently, trypsin was used throughout the remainder of the experiments.

Figure 3.05.A

GST Activity of Cytosols Prepared from Adrenocortical Cells on Different Days of Culture Using Different Cell Harvesting Methods.

The cytosolic fraction was prepared from adrenocortical cells maintained in primary culture (see "methods") and assayed for GST activity using CDNB as the substrate. Cytosols were prepared from cells on each of the first seven days of primary culture, using different methods for detaching the cells from the culture flasks. Initial experiments used a manual cell scraper, whilst later experiments used trypsin (0.01%) to detach the cells. The trypsinisation was seen to give higher values of GST activity without loss of activity to the harvesting medium. Consequently, this method was chosen for further experiments.

Figure 3.05.A

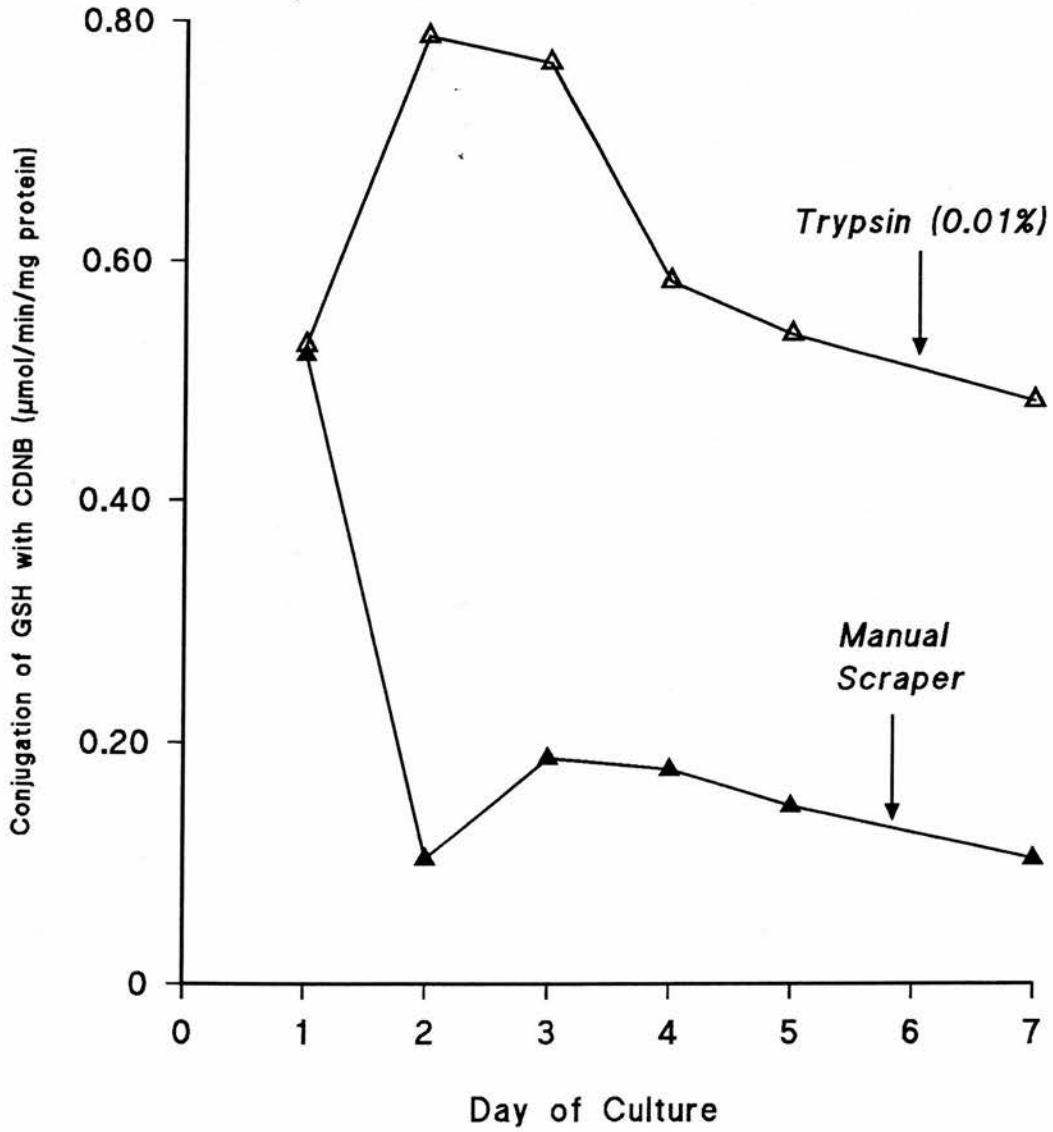

0.80
0.60
0.40
0.20
0
Conjugation of GSH with CDNB (µmol/min/mg protein)
Trypsin (0.01%)
Manual
Scraper
0
1
2
3
4
5
6
7
Day of Culture

Figure 3.05.B

Electrophoretic Analysis of Cytosols Prepared from Adrenocortical Cells in Primary Culture on Different Days of Culture.

The cytosolic fraction was prepared from adrenocortical cells which had been in primary culture for number of days specified, and analysed by SDS/PAGE. In addition to the use of rat liver GST markers, the GST pool from bovine adrenal cortex tissue purified on glutathione-Sepharose 6B was included in the gel, which was loaded as follows: lanes designated "M" contained a rat liver GST isoenzyme mixture; lanes 1 and 7, GST isoenzyme pool from bovine adrenal cortex purified on glutathione-Sepharose 6B (see figs 3.02.G-I); lane 2, cytosol from adrenocortical cells in suspension (day 1 of culture); lane 3, cytosol from adrenocortical cells on day 2 of culture; lane 4, cytosol from adrenocortical cells on day 3 of culture; lane 5, cytosol from adrenocortical cells on day 4 of culture; lane 6, cytosol from adrenocortical cells on day 7 of culture.

Figure **3.05.B**

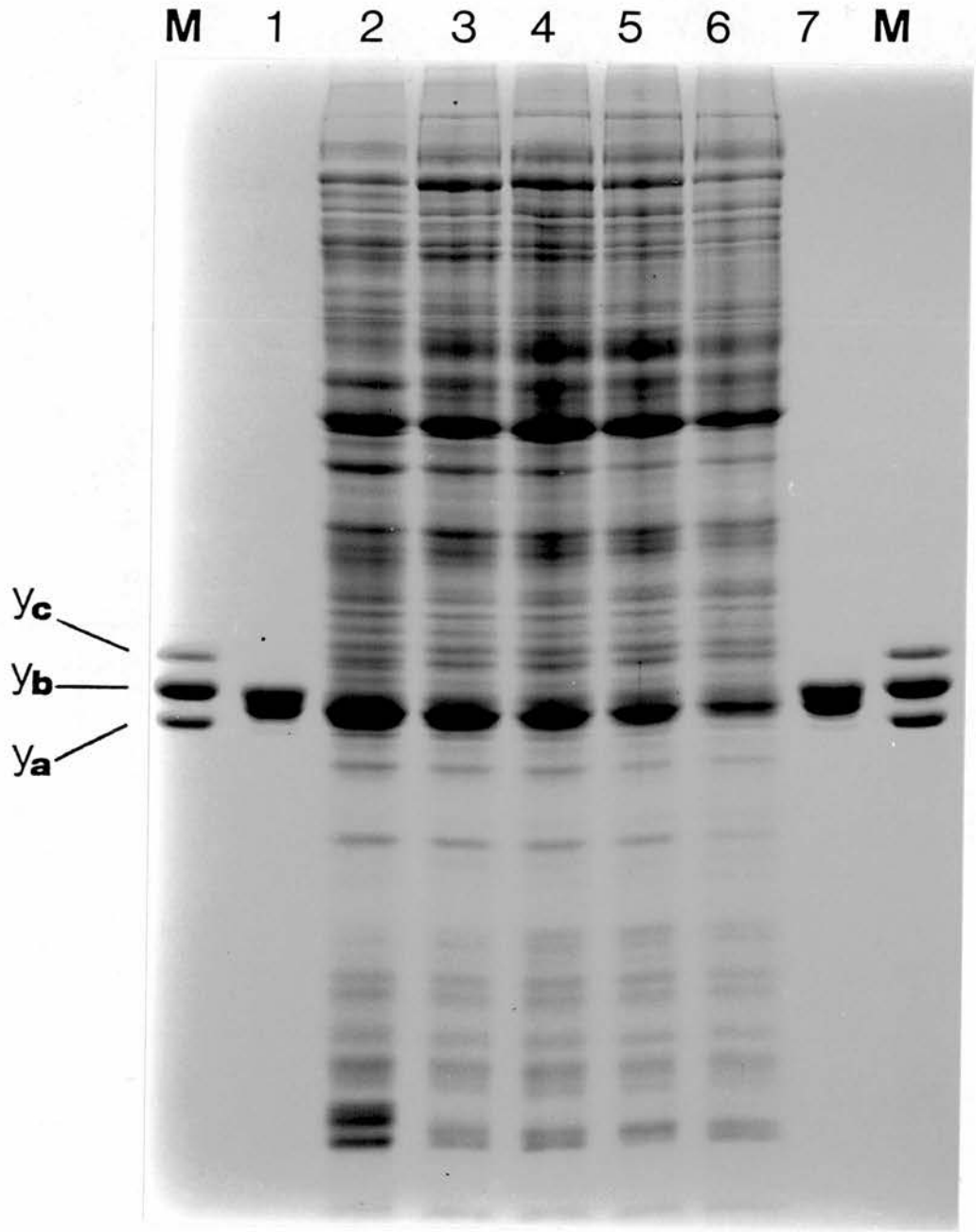
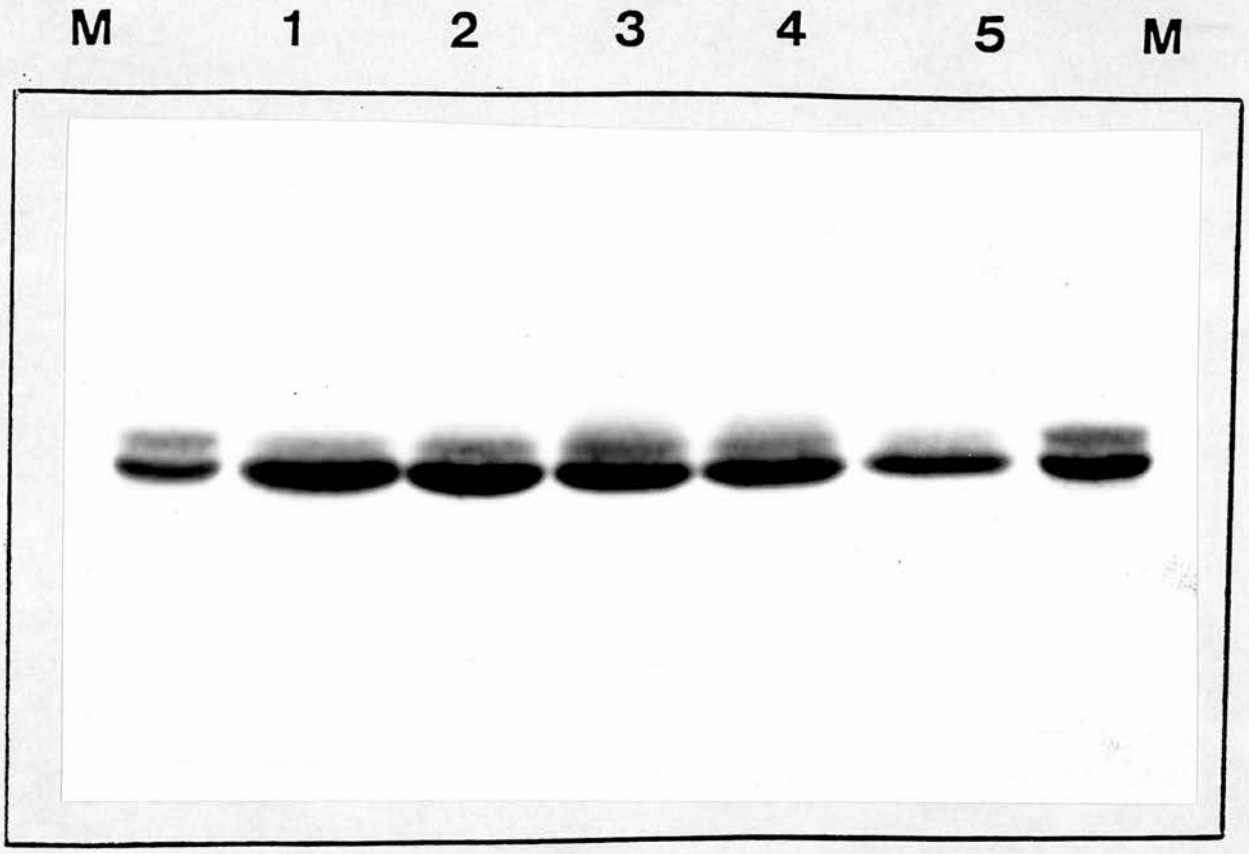


Figure **3.05.c**



Immunoblotting of Cytosols from Adrenocortical Cells on Different Days of Culture.

Immunoblotting was performed on cytosols from adrenocortical cells which had been in primary culture for the number of days specified, using the antibodies raised against the GST isoenzyme pool from bovine adrenal cortex previously purified on glutathione-Sepharose 6B. The loadings were as follows: lanes designated "M" contained cytosol prepared from whole adrenal cortex tissue (bovine); lanes 1, 2, 3, 4, and 5, cytosols prepared from adrenocortical cells in primary culture on days 1, 2, 3, 4, and 7 respectively.

Table 3.05(A)

Specific Activities of GSH-dependent Enzymes in Adrenocortical Cell Cytosols on Different Days of Culture Using a Range of GST Substrates.

Day of Culture	Specific Activity (μmol/min/mg protein)					
	CDNB	CuOOH	H_2O_2	ADD	NON	NPA
1	0.61	0.51	0.38	0.037	0.151	0.050
2	0.69	0.57	0.36	0.036	0.236	0.086
3	0.61	0.21	0.14	0.014	0.130	0.055
4	0.45	0.41	0.27	0.018	0.135	0.057
5	0.45	0.29	0.22	0.011	0.112	0.042
8	0.37	0.23	0.20	0.018	0.082	0.037
Tissue Cytosol	0.29	0.24	0.18	0.015	0.065	0.030

Abbreviations:

CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; H_2O_2, hydrogen peroxide; ADD, Δ^5androstene-3,17-dione; NON, 4-hydroxy-2-enal; NPA, p-nitrophenyl acetate.

In addition to using CDNB, several other model GST substrates were employed in the measurement of GST activity in adrenocortical cell cytosols on different days of primary culture (Table 3.05.A). As was the case for CDNB, the substrates 4-hydroxynon-2-enal and p-nitrophenyl acetate showed an increase in activity on day 2 of culture, which gradually decreased thereafter up to day 8 of culture. However, cumene hydroperoxide, hydrogen peroxide and Δ^5androstene-3,17-dione, generally maintained the same activities on the first two days of cutlure, which subsequently dropped to about half of the initial value on day 3. Activities with these substrates tended to show slight increases on day 4, which then decreased thereafter. Interestingly, for all substrates, specific activities for cells in culture (especially days 1 and 2) were markedly higher (2-3 fold) than cytosol prepared from whole adrenal cortex tissue (Table 3.05.A).

Analysis of cytosols from bovine adrenocortical cells on different days of primary culture by SDS/PAGE revealed the same abundant proteins in the GST region of the gel as whole adrenal cortex tissue cytosol (Fig. 3.05.B). Furthermore, in the absence of contamination from other cell types, this gel showed these proteins to be even more abundant in cytosols from adrenocortical cells in primary culture relative to the other cytosolic proteins. Indeed, the fast-moving alpha-class subunit binding to GSH-Ag appeared to be the major cytosolic protein. This gel also showed a slight decrease in levels of these proteins by day 7 of culture (lane 7, Fig. 3.05.B), which was given further support by the results of immunoblotting of these same cytosolic fractions (Fig. 3.05.C). Using the antibody raised against the alpha-class GST pool purified on GSH-Ag, there was a definite decrease in staining on the Western blot by day 7 of culture (lane 5), thus supporting the findings of GST activity measurements with a range of GST substrates.

(ii) Effects of Steroidogenic Agonists on GST Expression

The effects of the agonists, AII and ACTH, on GST expression in bovine adrenocortical cells in primary culture were investigated. These experiments were carried out with the prior knowledge that these agonists stimulate the biosynthesis and release of cortisol by bovine adrenocortical cells *in vitro*. The conditions employed in these experiments were based on observations made by other workers that day 3 cells in primary culture were found to give an optimal cortisol response to ACTH at 10^{-9} M. In the case of AII, day 4 cells are known to respond

Figure 3.05.D

Activities of GSH-Dependent Enzymes in Cytosols from Whole Adrenal Cortex Tissue and Adrenocortical Cells in Primary Culture, and Investigation of the Effect of Agonist Stimulation on Activities of These Enzymes in Primary Cultures Derived from Adrenocortical Cells.

Enzyme activities were measured for both whole adrenal cortex tissue cytosol and cytosol prepared from unstimulated cells in primary culture using 1-chloro-2,4-dinitrobenzene (CDNB), cumene hydroperoxide (CuOOH) and hydrogen peroxide (H_2O_2) as substrates. In addition, the same substrates were used to measure enzyme activities of cytosols prepared from adrenocortical cells in primary culture which had been stimulated with either ACTH (10^{-10} M, day 3 cells) or Angiotensin II (10^{-8} M, day 4 cells). The cells were exposed to these agonists for 24 hours, during which time the agonists were replaced several times (see text for details).

Figure 3.05.D

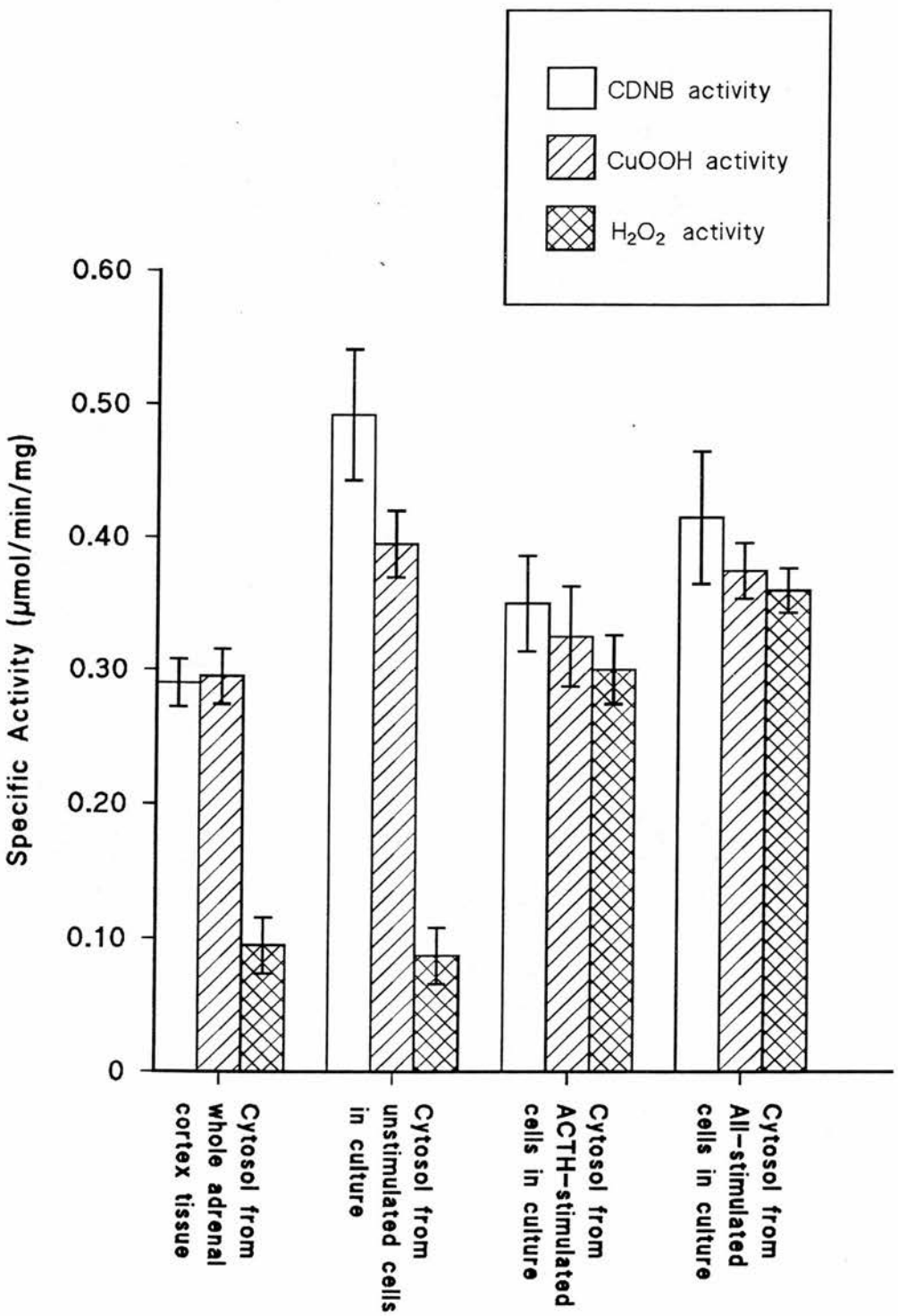
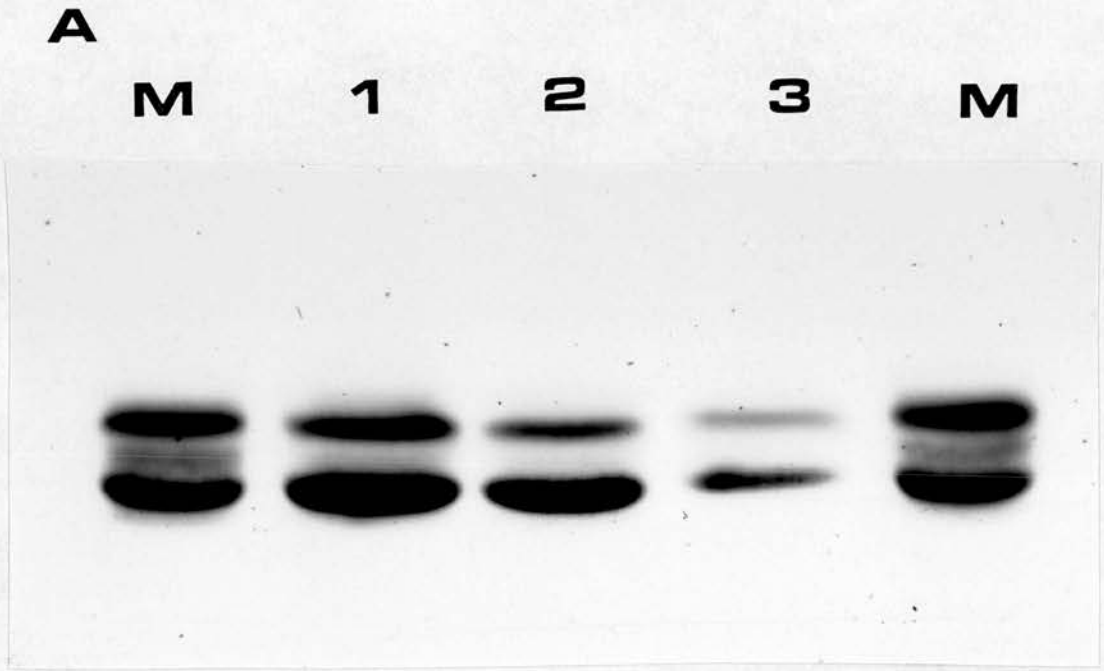

CDNB activity
CuOOH activity
H_2O_2 activity
Specific Activity (µmol/min/mg)
0.60
0.50
0.40
0.30
0.20
0.10
0
Cytosol from whole adrenal cortex tissue
Cytosol from unstimulated cells in culture
Cytosol from ACTH-stimulated cells in culture
Cytosol from All-stimulated cells in culture

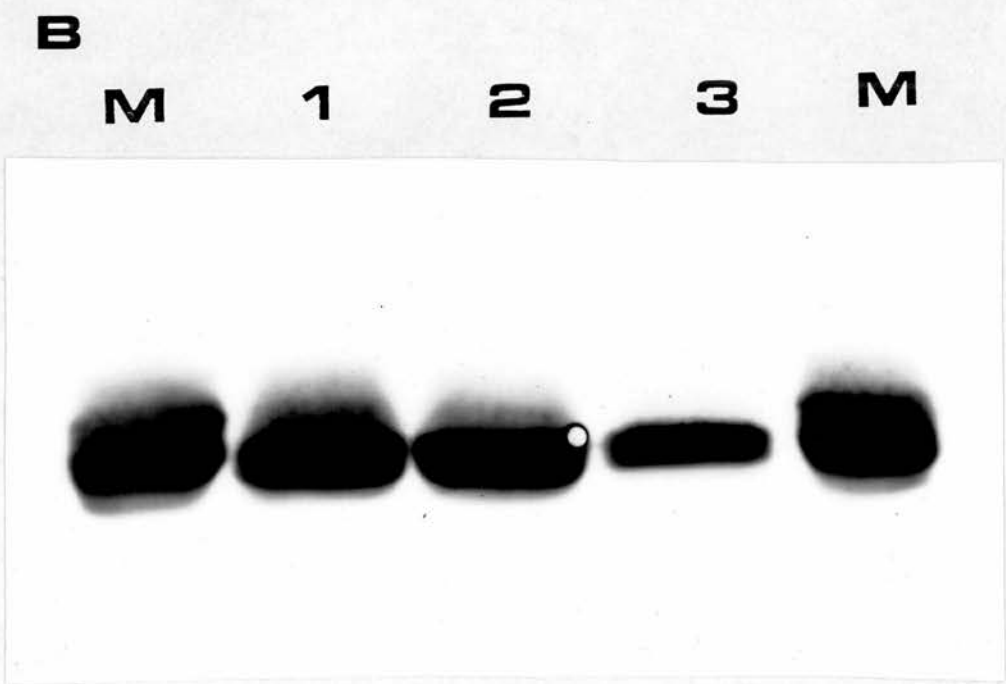
Figure 3.05.E

Immunoblotting of Cytosols from Adrenocortical Cells in Primary Culture Which Had Been Exposed to Either ACTH or Angiotensin II (AII).

Immunoblotting was carried out on the cytosolic fraction from adrenocortical cells which had been stimulated with either AII (10^{-8} M, day 4 cells) or ACTH (10^{-10} M, day 3 cells) for 24 h. Included for comparison were whole adrenal cortex tissue cytosols, as well as the cytosolic fraction from unstimulated adrenocortical cells on day 4 of primary culture. Blot **A** used antibodies that had been raised against the "slow-migrating" subunit purified from bovine adrenal cortex on reverse-phase hplc following affinity chromatography on S-Hexylglutathione-Sepharose 6B (peak 1, fig. 3.02.B); blot **B** used the antibodies that had been raised against the GST isoenzyme pool from bovine adrenal cortex that had been purified on glutathione-Sepharose 6B (see fig. 3.02.I). The loadings in both blots were as follows: lanes designated "M" contained whole adrenal cortex cytosol (bovine); lane 1, cytosol from adrenocortical cells on day 3 of culture; lane 2, cytosol from day 4 adrenocortical cells in culture that had been stimulated with AII (10^{-8}); lane 3, cytosol from day 3 adrenocortical cells in culture that had been stimulated with ACTH (10^{-10}).

Figure **3.05.E**





maximally to this agonist at 10^{-7} M. The final concentrations of agonists used were submaximal to avoid lengthy exposure (24 h) of the cells to the maximal concentration, with potentially toxic effects. The 24 h time period used for exposure of the cells to the agonists was chosen from the results of preliminary experiments (not shown) in which cells were exposed to 10^{-10} M ACTH for different time periods over a 48 h period.

The cytosolic fractions from adrenocortical cells stimulated with either agonist and from unstimulated cells ("control") were assayed for GST activity using either CDNB, cumene hydroperoxide (CuOOH) or hydrogen peroxide (H_2O_2) as substrates (Fig. 3.05.D). There was evidence that ACTH did cause a slight drop in GST activity using CDNB and CuOOH, although All did not show any significant change with these substrates. However, both agonists showed a 3-4 fold elevation in activity with H_20_2 as the substrate (i.e. selenium-dependent glutathione peroxidase). Immunoblotting using the anti(Ya_1) antiserum (as described earlier), or antiserum raised against the GSH-Ag purified alpha-class GST pool, supported these observations (blots A and B respectively, Fig. 3.05.E). Using the anti(Ya_1) antisera, ACTH appeared to down-regulate both GST bands (lane 3), whereas All showed only a slight down-regulatory effect (lane 2). Using the antibody raised against the GSH-Ag purified pool, ACTH again appeared to have a slight down-regulatory effect (lane 3, blot B), although All did not affect expression of this enzyme (lane 2). The implications of these results will be discussed in the next chapter.

Chapter 4: DISCUSSION.

DISCUSSION

Information regarding GST expression in the bovine species is limited, and initial experiments in this thesis entailed a study of the GST isoenzymes present in a number of bovine organs. Later work focused on the bovine adrenal cortex and characterisation of GST isoenzymes present in the cytosolic fraction. As a species comparison, the GSTs expressed by human adrenal cortex were also investigated. Finally, on the basis of observations made using whole adrenal cortex tissue cytosol, experiments were carried out to investigate the expression of alpha-class GSTs in primary cultures derived from bovine adrenocortical cells.

This final chapter will discuss the results obtained under the following sub-sections: (1) properties of bovine GSTs; (2) identification, classification and properties of GSTs in bovine adrenal cortex; (3) identification, classification and properties of GSTs in human adrenal cortex; (4) GST expression in bovine adrenocortical cells in primary culture.

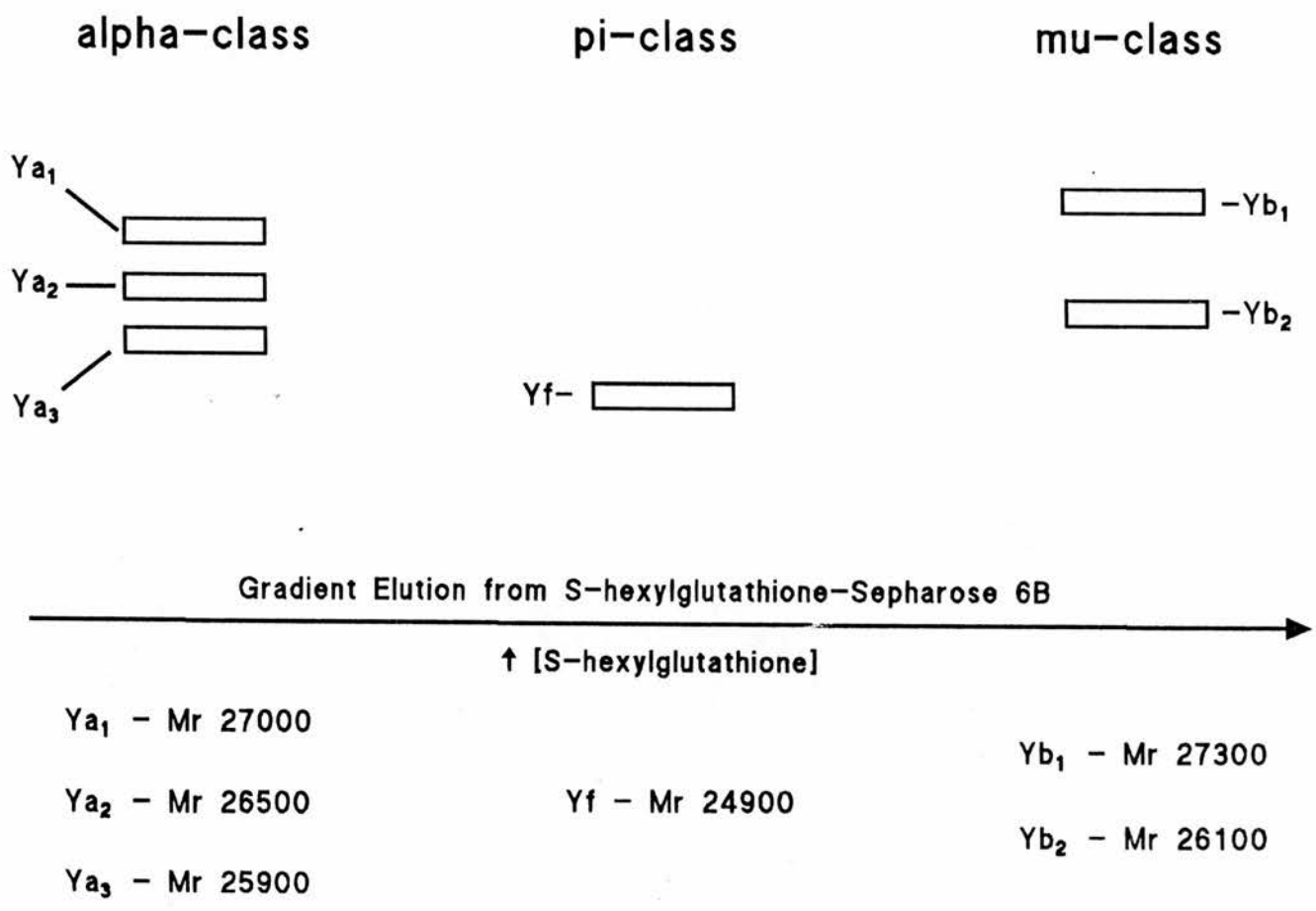
4.01 Properties of Bovine GSTs

Affinity chromatography on S-hexylglutathione-Sepharose 6B (S-hexG-Ag) was carried out to purify GSTs from the cytosolic fraction of a number of different bovine organs (see Section 3.01 of "Results"). For each organ, fractions were collected during the gradient elution with S-hexylglutathione and analysed for both protein content and GST activity (using both CDNB and CuOOH as substrates). SDS/PAGE revealed the presence of at least six GST subunits with different electrophoretic mobilities and elution positions during gradient elution on S-hexG-Ag: polypeptides of Mr 27 000, 26 500 and 25 900 eluting between 0.011-0.038 mM S-hexylglutathione; a polypeptide of Mr 24 900 eluting between 0.050-0.093 mM S-hexylglutathione; finally, two polypeptides of Mr 27 300 and 26 100 eluting between 0.100-0.200 mM S-hexylglutathione. Figure 4.01.A summarises the classification, elution positions and electrophoretic mobilities of these different polypeptides.

The GSTs which eluted at different positions on the gradient were further classified on the basis of results from the immunoblotting experiments (see Fig. 3.01.A (i)/(ii)) using antisera raised against different rat GST subunits (for details of specificity, see **Hayes & Mantle, 1986b).** In these

Figure 4.01.A

Diagrammatic Representation of Electrophoretic Migration Positions of Bovine GSTs



experiments, marked cross-reactivities were observed in lanes 1 and 2 using the anti-(rat Ya) antiserum (blot A, Fig. 3.01.A (ii)); lanes 3 and 4 using the anti-(rat Yf) antiserum (blot B, Fig. 3.01.A (ii)); lanes 5-8 using both anti-(rat Yb_1) and anti-(rat Yb_2) antisera (blots C and D respectively, Fig. 3.01.A (ii)). From these results it was possible to designate the subunits purified on S-hexG-Ag into the following GST classes: alpha-class GST, Ya_1 (Mr 27 000), Ya_2 (Mr 26 500) and Ya_3 (Mr 25 900); pi-class GST, Yf (Mr 24 900); mu-class GST, Yb_1 (Mr 27 300) and Yb_2 (Mr 26 100) [see Fig. 4.01.A for a summary of this information]. The "Ya" designation was adopted for the alpha-class GST because of the marked cross-reactivity of these alpha-class GSTs with the anti-(rat Ya) antiserum, although very little cross-reactivity with the anti-(rat Yc) antiserum (result not shown). The "Yb" nomenclature was adopted for the mu-class GST since these subunits cross-reacted with both anti-(rat Yb_1) and anti-(rat Yb_2) antisera although showed very little cross-reactivity with anti-(rat Yn) antiserum (result not shown). Interestingly, the anti-(rat Yb_1) antiserum showed a higher degree of cross-reactivity than the anti-(rat Yb_2) antiserum, especially with the faster-migrating subunit, whereas the anti-(rat Yb_2) antiserum cross-reacted moderately with both electrophoretic bands. The anti-(rat Yf) antiserum cross-reacted markedly with the fast-migrating bovine pi-class GST (Mr 24 900) which was consequently designated "Yf". This result was not surprising since the bovine pi-class GST showed a very similar electrophoretic mobility to the rat Yf subunit, unlike the other bovine GST classes which appeared to differ in mobility when compared to their immunochemically-related rat GST isoenzyme.

The expression of these multiple GST subunits by different bovine organs has been summarised in Table 4.01.A. For the alpha-class GSTs, both liver and testes appeared to express all 3 polypeptides, with the adrenal cortex showing a similar pattern of expression, apart from low levels of the Ya_2 GST. Interestingly, the lung expressed high levels of the Ya_1 GST with no evidence of either Ya_2 or Ya_3 GSTs. The electrophoretic data indicating alpha-class GST expression by these tissues was further supported by measurement of enzyme activities using CuOOH as the substrate (see elution profiles 3.01 B, D, F and H). Conversely, activity towards CuOOH could not be detected in the spleen, kidney or heart affinity-purified fractions, consistent with the lack of alpha-class GSTs in cytosols from these organs. All bovine organs examined,

Table 4.01.A

GSTs Expressed by Different Bovine Organs.

	Ya_1	Ya_2	Ya_3	Yf	Yb_1	Yb_2
adrenal cortex	+	+/−	+	+	+	+
liver	+	+	+	+/−	+	+
testes	+	+	+	+	+	+
lung	+	−	−	+	+	+
spleen	−	−	−	+	+	+
kidney	−	−	−	+	+	+
heart	−	−	−	+	+	+/−

The expression of multiple GST subunits by different bovine organs has been summarised on the basis of observations made during SDS/PAGE analysis. No attempt has been made to quantify expression levels of these GSTs, and the symbols + or − merely represent the presence or absence of each GST respectively. The designation +/− indicates that there may be trace amounts of the particular GST in the organ being studied.

except the liver, expressed the pi-class GST Yf subunit. This result is not surprising since livers from several other species (e.g. the rat) have been found to express low levels of pi-class GST (**Hayes & Mantle, 1986b**). The mu-class GSTs were found in all bovine organs studied, with the probable exception of heart cytosol which expressed only low levels of the Yb_2 subunit.

As expected from the studies of the rat GSTs, the bovine enzymes demonstrate a marked organ-specific expression. The tissue-specific expression of GST isoenzymes presumably reflects differences in metabolic functions carried out and the physiological stresses encountered by the different organs. The apparent universal expression of both pi- and mu-class GSTs in the organs studied may reflect a more general role for these enzymes, such as in the detoxification of exogenous compounds. However, the organ-specific expression of the alpha-class GST isoenzymes implies a function which is specific for those organs in which they are found (e.g. adrenal cortex, liver, testes and, to a lesser extent, lung). This organ-specific expression of alpha-class GSTs will be discussed in the next sub-section.

Classification of the bovine GST subunits was achieved on the basis of their immunochemical properties and mobilities during SDS/PAGE. The catalytic properties of the bovine GSTs (see Table 3.01.A) were compared with the specific activities of both rat and human GSTs (Tables 4.01.B and 4.01.C respectively), incorporating data from a number of sources (rat - **Hayes, 1986; Mannervik & Danielson, 1988;** human - **Stockman *et al.*, 1987**). Using CDNB as the substrate, those fractions containing the pi-class GST showed highest activity, with fractions containing both alpha- and mu-class GSTs displaying approximately one-half of the pi-class activity. The bovine GSTs exhibited considerably lower activity towards CDNB than the GST classes in the rat and human; this cannot be due to an inhibitory effect of S-hexylglutathione since all bovine GST pools were extensively dialysed against a neutral pH buffer before activity analysis. The bovine enzymes also had low activity towards ethacrynic acid as a substrate. Both rat and human pi-class enzymes show highest activity with this substrate, which was also true for those fractions containing the pi-class enzyme in the bovine species. However, as for CDNB, the values obtained for the bovine enzyme were substantially lower.

Table 4.01.B
Specific Activities of Rat Glutathione S-Transferases.

Substrate	class	Alpha			Mu			Pi
	subunit	Ya	Yc	Yk	Yb_1	Yb_2	Yn	Yf
1-Chloro-2,4-dinitrobenzene		50	17	10	58	17	190	24
Cumene hydroperoxide		3.1	7.9	1.1	0.35	0.72	0.19	0.048
Δ^5Androstene-3,17-dione		4.2	0.36	N.D.	0.02	0.002	N.D.	N.D.
Ethacrynic acid		0.08	1.24	7.0	0.08	0.62	0.057	3.84
p-Nitrophenyl acetate		1.32	0.58	0.09	0.18	0.23	0.05	N.D.
p-Nitrobenzyl chloride		1.5	0.3	1.1	14.0	14.4	1.7	N.D.
1,2-Dichloro-4-nitrobenzene		>0.04	>0.04	0.12	5.3	0.18	2.85	0.048
trans -4-Phenyl-3-buten-2-one		>0.04	>0.04	0.10	0.05	1.18	0.019	0.22

Adapted from Mannervik & Danielson (1988); Hayes (1986)

N.D. denotes values which have not been determined.

Table 4.01.C
Specific Activities of Human GST Enzymes.

Substrate	Specific Activity (μmol/min/mg protein)				
	B_1B_1	B_1B_2	B_2B_2	μ	λ
1-Chloro-2,4-dinitrobenzene	82	117	80	272	212
Cumene hydroperoxide	31	92	104	N.D.	0.11
Ethacrynic Acid	0.11	0.16	0.14	0.22	1.22
p-Nitrophenyl acetate	0.66	0.97	0.24	N.D.	0.38
1,2-Dichloro-4-nitrobenzene	0.25	0.86	0.79	0	0.14
trans -4-Phenyl-3-buten-2-one	0	0	0	0.45	0.02

Adapted from Stockman *et al.*, (1987)

N.D. denotes values not determined.

Activities with the substrates Δ^5-androstene-3,17-dione, cumene hydroperoxide and 4-hydroxynon-2-enal were highest with those fractions containing alpha-class GSTs. Values with Δ^5-androstene-3,17-dione (Table 4.01.B) and 4-hydroxynonenal (see **Danielson *et al.*, 1987)** were considerably lower with the bovine GSTs; activity towards cumene hydroperoxide was almost equivalent to the rat, although much lower than the human enzymes. The substrate, p-nitrophenyl acetate, is most active with certain alpha-class GSTs in both the rat and human species, although both alpha- and mu-classes of bovine GST were found to be active with this substrate.

The bovine mu-class GSTs showed some activity with p-nitrobenzyl chloride, a substrate known to show high activity with both the rat Yb_1 and Yb_2 subunits. This substrate was the only one which appeared to show any specificity for bovine mu-class enzymes, since bovine mu-class enzymes failed to show significant activity with both 1,2-dichloro-4-benzene and *trans*-4-phenyl-3-butene-2-one (which show high activities with rat Yb_1 and Yb_2 subunits respectively). However, p-nitrobenzyl chloride was less effective as a substrate as compared to the rat. Thus, the bovine mu-class enzymes are catalytically distinct from those in the rat and human species that have been described to date.

In summary, the data suggest that the bovine GSTs show a different substrate specificity as compared to other species such as the rat and man. Considerable differences in specific activities using different model GST substrates were obtained, and collectively these observations illustrate the marked species differences existing for GSTs. The mobilities of the different bovine GST classes on SDS/PAGE differed from those of other species, and immunoblotting using various anti-(rat GST) antisera revealed varying degrees of cross-reactivity with the different bovine GSTs. Future studies would aim to provide homogeneous preparations of individual bovine GST subunits, with the subsequent determination of primary structure.

4.02 Identification, Classification and Properties of GSTs in Bovine Adrenal Cortex.

Following on from the study of GSTs in different bovine organs, the affinity-purified GSTs from adrenal cortex cytosol were further characterised. During the course of this work a major alpha-class GST was identified which did not bind to the S-hexG-Ag affinity matrix used in the first purification step, but was isolated using glutathione-Sepharose 6B (GSH-Ag). Subsequent experiments established that this phenomenon was not unique to the adrenal cortex, and a similar alpha-class GST could be purified from bovine liver and testes. This section will thus be subdivided into the following parts: (A) analysis of alpha-class GSTs purified from bovine adrenal cortex using S-hexylglutathione-Sepharose 6B (S-hexG-Ag); (B) identification and characterisation of the major alpha-class GST from bovine adrenal cortex; (C) tissue-specific expression of alpha-class GSTs in other bovine tissues.

(A) Analysis of alpha-class GSTs purified from bovine adrenal cortex on S-hexylglutathione-Sepharose 6B (S-hexG-Ag).

Before further chromatography was employed to resolve individual GST isoenzymes in the affinity-purified GST pools from bovine adrenal cortex, attempts were made to determine the isoelectric points of these proteins (Fig. 3.02.A). Results from isoelectric focusing revealed the alpha-class GST pool to consist of basic proteins with an approximate pI of 8.75, the pi-class GST pool to consist of mainly near-neutral proteins with an approximate pI of 7.30, and finally the mu-class GST pool to consist mainly of proteins with pI's between 7.80 and 7.90. These values are similar to those observed for rat and human isoenzymes (for specific values, see the following references: rat- **Mannervik & Jensson, 1982**; human, **Vander Jagt *et al.*, 1985**), with the exception of the higher pI value of the bovine pi-class isoenzyme. Furthermore, the bovine GSTs appear to display similar pI values to those observed for the mouse (see **Hayes *et al.*, 1987**), with the alpha-class enzymes appearing very basic (pI above 9.0), and the other two classes having pI values in the range 7.80-8.60.

Using this information, anion-exchange chromatography was employed to purify the mu/pi-class GST pool using a buffer adjusted to pH 8.9. (Fig. 3.02.D). This method produced a mixture

of both partially and fully purified GST fractions which were designated peaks 1-4 according to their order of elution. Peaks 1 and 2 appeared to contain pi-class GST whereas peaks 3 and 4 contained mu-class GST. Application of peaks 1 to 4 from the mono-Q anion-exchange column to a μBondapak reverse-phase h.p.l.c. column resulted in the resolution of four main protein elution peaks named a-d according to their order of elution (Fig. 3.02.E (i)-(iv)). SDS/PAGE analysis of the proteins collected from peaks a and b during reverse-phase h.p.l.c. suggested a very slight difference in electrophoretic mobility, which raises the question as to the number of Yf subunits making up the pi-class GST family in this species. As described during the introduction to this thesis, **Bora *et al*. (1989)** demonstrated a marked sequence homology between the human heart Yf subunit and the major fatty-acid ethyl ester synthetase in this organ. The two enzymes were also shown to display interchangeable activities and these findings, in addition to the identification of a possible second pi-class GST gene by **Board *et al*** in 1989, collectively support the existence *in vivo* of two (or more) Yf subunits. In the case of bovine adrenal cortex, further analysis would be required to ascertain whether the two reverse-phase h.p.l.c. peaks were due to different polypeptides or merely interchangeable forms of the same protein. Unfortunately, activity analyses were not possible due to the denaturing effect of reverse-phase h.p.l.c., although primary sequence information would be invaluable here.

SDS/PAGE analysis of peaks c and d obtained during reverse-phase h.p.l.c. analysis of the mu-class enzymes purified during anion-exchange chromatography (peaks 3 and 4, Fig. 3.02.D) revealed each peak to contain different proteins (Fig. 3.02.F). Peak 3 consisted entirely of the Yb_2 (Mr 26 100) polypeptide defined earlier in this chapter, with peak 4 consisting of almost equimolar amounts of both Yb_1 and Yb_2 polypeptides. It remains unclear why the Yb_1 subunit should be resolved from the Yb_2 subunit (and not *vice versa*) using this method, although it should be noted that the faster-migrating band observed in peak 4 may be different from the polypeptide found in peak 3. Again, primary sequence information would prove most useful in clarifying the exact number of distinct mu-class enzymes expressed by bovine adrenal cortex.

Ion-exchange chromatography proved to be an unsuccessful method for resolving the alpha-class GSTs partially purified on S-hexG-Ag: different buffers over a range of pH's using

both anion and cation exchange columns were employed, but failed to resolve the two main polypeptides observed on SDS/PAGE. Consequently, aliquots of the alpha-class GST purified on S-hexG-Ag were applied directly to a μBondapak reverse-phase h.p.l.c. column. This matrix resolved two polypeptides (Fig. 3.02.B) which were named Ya_1 and Ya_3. These purified proteins were used to raise antisera which proved to be useful in later experiments, with the anti-Ya_3 antiserum proving to be specific for this subunit, but the anti-Ya_1 antiserum showing cross-reactivity with both Ya_1 and Ya_3 subunits.

In summary, the use of anion-exchange chromatography to further resolve bovine adrenal cortex GSTs partially purified on S-hexA-Ag, followed by reverse-phase h.p.l.c., proved to be an effective method of obtaining highly purified pi- and mu-class GSTs from this organ. Using this approach, there appeared to be at least one (and possibly two) distinct pi-class GSTs, and at least two (and possibly three) mu-class GST subunits in bovine adrenal cortex cytosol. Ion-exchange chromatography failed to resolve the alpha-class GSTs bound to S-hexG-Ag.

(B) Identification and characterisation of the major alpha-class GST from bovine adrenal cortex

Investigations into the GST isoenzymes in different bovine organs have primarily employed S-hexG-Ag as the affinity matrix for purification. However, analysis of the flow-through from this column revealed that about 35% of GST activity towards CDNB did not bind to S-hexG-Ag. Failure to bind to S-hexG-Ag has also been observed for the rat alpha-class Yk subunit **(Hayes, 1986)**, and since then several other GST subunits have been found to show very low affinity for S-hexG-Ag **(Hayes, 1988).**

Affinity chromatography of this S-hexG-Ag flow-through fraction on glutathione-Sepharose 6B (GSH-Ag) resulted in the purification of abundant GST enzyme activity (comprising approximately 1.3% of the total cytosolic protein) which comprised two distinct electrophoretic bands by SDS/PAGE (Mr 25 900 and 26 500) and demonstrated many characteristics of alpha-class GSTs. The polypeptide of Mr 25 900 was thought to be equivalent to the Ya_3 subunit purified on S-hexG-Ag. However, the polypeptide of Mr 26 500 showed a different electrophoretic

mobility to the S-hexG-Ag purified alpha-class GST subunits (Ya_1 and Ya_3) and was consequently named Ya_2.

Besides the sheer abundance of this purified enzyme pool, of particular interest were the marked selenium-independent glutathione peroxidase and Δ^5-ketosteroid isomerase activities exhibited by this enzyme, as well as the relatively high activity with 4-hydroxynonenal. Activity against these substrates was considerably higher than for the alpha-class GSTs purified on S-hexG-Ag and may therefore implicate the GSH-Ag purified enzymes in the normal metabolism of the adrenal cortex. As described in the introduction to this thesis, lipid peroxidation and other aspects of oxygen toxicity are a potential problem for adrenocortical tissue, and the presence of a high peroxidase activity to overcome such oxidative stress would be appropriate. A possible function *in vivo* for these alpha-class GSTs may be, therefore, to prevent adrenocortical cell damage caused by lipid hydroperoxides and other free oxygen radicals formed as by-products during the synthesis of steroids.

The relatively high activity of the GSH-Ag purified enzyme towards 4-hydroxynon-2-enal lends further support to such a function. The 4-hydroxyalkenals are known to occur in the cell as a result of oxidative metabolism of endogenous, as well as foreign, compounds and studies have shown relatively large amounts to be produced during stimulated lipid peroxidation **(Esterbauer *et al.*, 1982; Poli *et al.*, 1985).** The relatively high activity of the bovine alpha class enzymes towards 4-hydroxynon-2-enal thus supports the idea that they might be required for the detoxification of endogenous oxidised products formed during steroidogenesis. The high levels of reduced glutathione (and selenium) known to exist in the bovine adrenal cortex **(Hornsby & Crivello, 1983b)** lend further support for this function. However, it should be noted that the bovine adrenal cortex is known to be remarkably well-served by a number of biological antioxidants (as reviewed by **Hornsby & Crivello, 1983b)**, and the role of alpha-class GSTs may only be as part of an overall antioxidant system.

Alternatively, the marked Δ^5-ketosteroid isomerase activity exhibited by the alpha-class GSTs purified on GSH-Ag suggests another possible function. This isomerisation reaction occurs at a number of stages during the synthesis of steroids (see Fig. 1g), where it is assigned to the

microsomal 3-β-steroid dehydrogenase/ Δ^5-ketosteroid isomerase enzyme **(Naville *et al.*, 1991)**. The relatively high isomerase activity shown by the bovine alpha-class enzyme(s) might therefore serve a function in the steroid biosynthetic pathway, though how this activity would relate to the Δ^5-ketosteroid isomerase activity shown by the microsomal enzyme is unclear. This clearly remains speculative at this stage and further experiments would be required to test the substrate specificity and kinetic characteristics of this reaction in more detail to help answer this question.

Further attempts were made to resolve the two polypeptides present in the GSH-Ag enzyme pool, designated Ya_2 and Ya_3, with a view to determining the substrate specificities of these subunits. Application of the GSH-Ag purified pool to an anion-exchange column resulted in a complex pattern of at least six protein peaks eluting from this column (Fig. 3.02.J) which consisted of both homogeneous and heterogeneous fractions (Fig. 3.02.K). Determination of activities of individual fractions using CDNB, CuOOH, Δ^5-androstene-3,17-dione and 4-hydroxynonenal as substrates revealed an even more complex situation, with the various protein peaks showing different levels of activity with different substrates. To clarify the situation and enable an estimation of the number of distinct alpha-class GST subunits present in bovine adrenal cortex, the activity data obtained from the anion-exchange chromatography step (Figs. 3.02.I (i-iv)) has been summarised in Table 4.02.A. Although the elution of multiple peaks during ion-exchange chromatography does not necessarily mean that there are a correspondingly large number of different isoenzymes, activity analysis of each fraction collected during chromatography revealed that each peak obtained displayed a different level of activity with each of the substrates (Table 4.02.A). Not surprisingly, the protein collected from peak 1 was active with all substrates since this pool was found to consist of at least 2 polypeptides during SDS/PAGE analysis (see Fig. 3.02.K). As indicated in Table 4.02.A, peak 2 could be split into two further peaks on the basis of GST activity (**a** and **b**, as indicated). These two peaks show quite different levels of activity with the different substrates and suggest them to cosist of different GST subunits. The range of activities obtained for each of the peaks collected during gradient elution is complex and,

Table 4.02.A

Summary of GST Activities Using Different Model Substrates of the Peaks Obtained During Anion–Exchange Chromatography of GSH–Ag Purified Isoenzymes from Bovine Adrenal Cortex.

Substrate	Peak Number						
	1	2a	2b	3	4	5	6
CDNB	+	+	+/–	+	+	+/–	+
CuOOH	+	+	–	+	–	+	+
ADD	+	+	–	+	–	–	–
OH–NON	+	+/–	+	+/–	+	–	–

Abbreviations:

+ denotes high activity; +/–, some activity; –, no activity.

CDNB, 1–chloro–2,4–dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5Androstene–3,17–dione; OH–NON, 4–hydroxynonenal.

as the fractions making up these peaks appeared homogeneous during SDS/PAGE (Fig. 3.02.K), these data suggest the presence of at least 3 distinct GST subunits in the GSH-Ag pool.

Unfortunately, the simultaneous application of different fractions making up each of the six peaks resulting from anion-exchange chromatography (Fig. 3.02.J) to a reverse-phase hplc system failed to give any further resolution (results not included). Overall, it is only possible to speculate that there are at least 3 different alpha-class GST subunits present in the GSH-Ag purified pool, of which two have been named Ya_2 and Ya_3. In summary it is possible to speculate further that, including the Ya_1 subunit purified on S-hexG-Ag only, there are at least 4 alpha-class GST subunits in bovine adrenal cortex cytosol. However, further experiments are clearly required to verify this.

(C) Tissue-specific expression of alpha-class GSTs in other bovine organs

Further experiments were carried out to investigate the possibility that other bovine organs may also express similar GST isoenzymes. Initial observations using SDS/PAGE analysis of the cytosolic fractions prepared from different bovine organs revealed differences in GST expression between the different zones of the adrenal cortex (Fig. 3.03.A). The zona fasciculata layer of the adrenal cortex appeared to express even higher levels of GST than whole tissue cytosol, whilst the zona glomerulosa cytosol appeared to express reduced levels of the slower-migrating GSTs. These observations are especially interesting in the context of known specific functional differences between these two zones of the adrenal cortex, with the zona fasciculata being the site of glucocorticoid synthesis (predominantly cortisol in man and bovine species) and the zona glomerulosa the site of mineralocorticoid synthesis (predominantly aldosterone). Immunohistochemical analysis of bovine adrenal cortex sections using antisera raised against each of the bovine alpha-class GSTs would prove to be very useful in confirming such zonal differences.

These observations are also interesting in the context of sex steroid production from the zona fasciculata, since in this zone androgen production will be high relative to the other zones. As mentioned earlier, the alpha-class GST pool purified from bovine adrenal cortex on GSH-Ag

displays marked Δ^5-ketosteroid isomerase activity. It would therefore be of interest to determine whether a similar affinity-purified pool from the zona fasciculata would retain a significantly higher Δ^5-ketosteroid isomerase activity as compared with the same pool purified from the zona glomerulosa. Such results would consequently give a clearer indication of whether the Δ^5-ketosteroid isomerase activity displayed by the GSH-Ag purified pool has any significant physiological function, as discussed earlier.

The results of immunoblotting further confirmed the observations made during SDS/PAGE analysis. Blots using anti-(bovine Ya_1) antiserum revealed marked cross-reactivities with cytosols from all sections of the adrenal gland, testes, liver and lung as expected. Significantly, this blot confirmed the higher levels of Ya_1 and Ya_2 subunits in the zona fasciculata cytosol compared to the zona glomerulosa, as discussed above. The anti-(bovine Ya_3) antisera showed similar crossreactivities (with the exception of the lung), again emphasising the difference between the two zones of the adrenal cortex. Antisera raised against the bovine alpha-class GST pool purified on GSH-Ag showed the same pattern of cross-reactivity as the anti-(Ya_3) antisera, although there was more cross-reactivity with the Ya_2 subunit.

Both liver and testes thus appear to display a similar high level of expression of alpha-class GSTs as the adrenal cortex; the moderate levels of cross-reactivity found in the adrenal medulla cytosol is probably not significant and is likely to reflect contamination from the adrenal cortex, estimated to be approximately 10% of the medulla preparation. These results were given support from observations made during routine analysis of cytosols from different bovine organs at different stages of affinity chromatography. Apart from the adrenal cortex, the only bovine organs displaying significant GST activity in the flow-through fraction from the S-hexG-Ag column were the liver and testes. Following application of S-hexG-Ag flow-through from these organs to GSH-Ag, almost all GST activity was removed. Subsequent elution of the GSTs bound to the GSH-Ag affinity matrix resulted in the purification of GST subunits from both organs which were almost identical in electrophoretic mobility to those purified from the adrenal cortex, consisting of two main polypeptides of approximate Mr 26 500 and 25 900 (see Figs. 3.03.D and 3.03.F).

Activity assays of the purified fractions using CuOOH provided further evidence that these purified enzymes consist of alpha-class GSTs. Notably, the purified liver enzymes were considerably more abundant than the equivalent enzymes in the adrenal cortex, whereas the purified testes enzymes showed values which were comparable with the adrenal cortex enzymes.

In summary, the abundant expression of alpha-class GSTs was not unique to the bovine adrenal cortex since cytosols from both the liver and testes were found to express high levels of the same enzymes. It is interesting that these organs are all capable of metabolising steroids, and lends support to the hypotheseis that the abundant expression of certain alpha-class GSTs may be a feature of steroid-metabolising organs.

(3) Identification, Classification and Properties of GSTs in Human Adrenal Cortex

Following the identification of the GSH-Ag bound alpha-class GST in bovine adrenal cortex, liver and testes, experiments were carried out to investigate alpha-class GST expression in man. The rat adrenal cortex is known to express relatively high levels of the alpha-class Yc subunit **(Sierakowski & Kraus, 1984)**, and the human adrenal cortex was chosen as an alternative species for investigating GST expression due to the limited knowledge of the GSTs expressed in human adrenocortical tissue.

Preliminary analysis of human adrenal cortex cytosol by SDS/PAGE revealed an abundant protein which appeared to co-migrate with the fast-migrating Ya_3 subunit expressed in bovine adrenal cortex cytosol (Fig. 3.04.A). Human adrenal cortex cytosol was also compared with human liver cytosol. This liver is known to express relatively large amounts of alpha-class GST **(Stockman *et al.*, 1985; 1987)** and, significantly, human adrenal cortex appeared to express similar levels of a corresponding protein in the same region of an SDS/PAGE gel as the liver alpha-class GST (Fig. 3.04.B). This protein, in addition to migrating in a similar position to the Ya_3 subunit described previously in bovine adrenal cortex cytosol, also showed a very similar electrophoretic mobility to the human liver GST B_1B_1 marker enzyme. Purification of human adrenal cortex GSTs using an approach identical to the bovine organ, resulted in the successful

purification of all GST activity on S-hexG-Ag, with none remaining in the flow-through fraction from this column (Table 3.04.A; Fig. 3.04.C). As for the bovine adrenal cortex GSTs purified on GSH-Ag, the purified human enzymes showed many characteristics of alpha-class GSTs, such as activity with the relevant model GST substrates and a similar electrophoretic mobility to the human alpha-class GST B_1 subunit (see Fig. 3.04.D). The isomerase activity of the purified human adrenal cortex GST was particularly noteworthy since existing information concerning the activity of human alpha-class GSTs towards Δ^5 androstene-3,17-dione is limited. The human liver GST B_1B_1 enzyme was found to exhibit high activity with this substrate (see Table 3.04.A) whereas the human liver GST B_2B_2 enzyme showed very little isomerase activity (result not included). These observations (in addition to helping identify the human adrenal cortex GST) are noteworthy from the point of view that the B_1 and B_2 subunits have been reported by **Hayes *et al.*, (1989a)** to differ by only 7 out of a total 221 amino acid residues, yet this study has shown marked differences in their activities towards Δ^5 androstene-3,17-dione.

The activities of the purified human enzymes towards a variety of substrates were very similar to the activities already documented for human liver GST B_1B_1 against the same substrates. Comparison of elution positions of the human adrenal cortex GST with human liver GST B_1B_2 standards supported the hypothesis that the main GST in human adrenal cortex is equivalent to the B_1 subunit (Fig. 3.04.E). Importantly, the GST pool purified on S-hexG-Ag from human adrenal cortex cytosol produced only one main peak during reverse-phase h.p.l.c., suggesting this human organ to contain mainly alpha-class GSTs, with very minor expression levels of the other GST classes. However, some degree of caution is required when making such generalisations about human GSTs: these enzymes are known to show polymorphic expression and, ideally, similar experiments would be required to be carried out on cytosols prepared from several other human adrenal cortex tissues.

As described above, the human adrenal cortex alpha-class GST was purified on S-hexG-Ag and not GSH-Ag as for the bovine enzymes. The reason for such a marked difference in binding affinity to S-hexG-Ag is unclear but suggests that structural differences do exist between the alpha-class enzymes from the two species, even though their catalytic properties are very

similar. An obvious difference between the alpha-class enzymes from both species is the presence of an additional, slower-migrating polypeptide (Mr 26 500) observed during SDS/PAGE analysis of the bovine enzyme. The corresponding electrophoretic band is only marginally visible during SDS/PAGE analysis of bovine alpha-class GSTs purified on S-hexG-Ag. In this regard, it is similar to the rat Yk subunit **(Hayes, 1986)** which also demonstrates a lack of affinity for S-hexG-Ag. This does not, however, explain the apparent lack of affinity of the faster-migrating polypeptide (Mr 25 900) towards S-hexG-Ag, and the question of whether this subunit is the same as the S-hexG-Ag purified Ya_3 subunit remains unclear. The affinity of the human alpha-class GST for S-hexG-Ag, however, could be attributed to species differences between the bovine and human alpha-class enzymes. It is also possible to speculate here that the slow-migrating electrophoretic band observed for the bovine alpha-class GSTs purified on GSH-Ag may be responsible for the high activity obtained using 4-hydroxynonenal. This is based on the observations that the single GST band from the human organ displayed both marked peroxidase and isomerase activities (i.e. greater than the bovine enzymes), yet the human liver GST B_1B_1 enzyme has a relatively low activity towards 4-hydroxynonenal **(Danielson *et al.*, 1987)**.

In summary, human adrenal cortex cytosol was found to express high levels of an alpha-class GST which appeared to be equivalent to the human liver GST B_1B_1. Previous studies involving extrahepatic human GSTs have suggested high levels of expression of a basic GST in the adrenal cortex **(Faulder *et al.*, 1987; Corrigall & Kirsch, 1988)**. However, this study is the first time that the alpha-class GST has been formally identified. Like the bovine alpha-class GSTs, levels of expression of this GST class were much higher than those of the mu- and pi-classes, displaying marked selenium-independent glutathione peroxidase and Δ^5-3-ketosteroid isomerase activities. The significance of these activities in the context of adrenocortical function have been discussed for the bovine enzymes, and the same reasoning would apply to expression of these enzymes in human adrenal cortex.

(4) Alpha-Class GST Expression in Adrenocortical Cells in Primary Culture

Having shown adrenal cortex cytosol to contain high levels of alpha-class GST, an initial study was undertaken to investigate the possible regulation of expression of alpha-class GST in primary cultures of inner zone bovine adrenocortical cells. Methodological problems were encountered in the harvesting of cells from culture flasks, although 0.01% trypsin was eventually found to detach cells with minimal leakage of cytosolic contents (see Fig. 3.05.A). Before detailed investigations were carried out on the expression levels of alpha-class GSTs in response to steroidogenic agonists, a day-by-day study of expression in unstimulated cells was undertaken in order to assess the effects of prolonged culture. Using a range of model GST substrates, the general pattern of activity revealed an increase on day 2 of culture which gradually decreased thereafter (see Table 3.05.A for details). It was decided to use cells on days 3 and 4 when GST expression was close to maximal. This time also appeared appropriate because steroidogenic agonists such as ACTH and angiotensin II (AII) show maximal stimulation of cortisol secretion from bovine adrenocortical cells on days 3 and 4 (**Williams *et al.*, 1989**).

Specific activities for GST activity measurements in cytosol from adrenocortical cells either in suspension (day 1) or in primary culture were significantly higher than cytosol from whole adrenal cortex tissue. This undoubtedly reflects the high purity of the zona fasciculata/ reticularis cell preparations compared to whole tissue (where contamination by blood and other cell types is likely). As described earlier, zona fasciculata/reticularis cytosol showed even higher levels of alpha-class GSTs as compared to whole adrenal cortex cytosol, which would be expected to be reflected by a similar increase of enzyme activities. The abundance of alpha-class GSTs in zona fasciculata/reticularis cytosol was particularly obvious on SDS/PAGE analysis of cytosols prepared from cultured bovine adrenocortical cells which showed the main alpha-class GST bands to stain more intensely than any other cytosolic protein (Fig. 3.05.B). This is not the case in gels which used whole adrenal cortex cytosol where extracellular proteins such as bovine serum albumin and haem are more abundant.

SDS/PAGE analysis of cytosols from bovine adrenocortical cells on different days of culture revealed, as expected from the activity data, a corresponding decrease in the levels of

proteins in the GST region. Immunoblotting using antisera raised against the bovine alpha-class GSTs purified on GSH-Ag confirmed these observations, indicating a decrease in expression by day 7 of culture (Fig. 3.05.D). One possible explanation for these findings could be that the lack of external regulatory factors, such as ACTH, causes levels of alpha-class GSTs to decrease. However, such apparent decreases may also be due to an aging effect whereby the cells in culture lose their ability to synthesise protein.

Some clue as to the answer was obtained from the results of experiments which investigated the effect of steroidogenic agonists such as ACTH and AII on alpha-class GST expression. Using CDNB as substrate, ACTH caused a significant decrease in GST activity, although alpha-class GST activity was not significantly affected (Fig. 3.05.D). Incubation of cells with AII did not have any significant effect on either CDNB or CuOOH activities. However, the selenium-dependent glutathione peroxidase activity did show a marked increase for both agonists, suggesting an important function *in vivo* for this enzyme during steroid production in adrenocortical cells. For example, this enzyme might be required for the endogenous detoxification of potentially toxic hydroperoxides formed during steroid biosynthesis, as discussed earlier.

Immunoblotting was carried out in an attempt to correlate the observed decrease in CDNB activity during ACTH stimulation. As expected, no changes in GST levels were observed in blots of cytosols from cells which had been stimulated with AII. However, a decrease was observed in cytosol prepared from ACTH-stimulated cells using antisera raised against bovine alpha-class GSTs, especially using the anti-(bovine Ya_1) antiserum. Interestingly, no difference in activity was obtained for cytosols from ACTH-stimulated cells using CuOOH as the substrate (unlike CDNB). This result is surprising since the changes observed during immunoblotting for the mass of the enzyme should have been sufficient to enable similar changes in activity to be detected.

The effects observed for ACTH do not correlate with those obtained for the day-by-day study. Prolonged culture (i.e. increased time without exposure to naturally-occurring steroidogenic agonists such as ACTH) had the effect of decreasing enzyme expression levels, an effect which was paradoxically also observed during ACTH stimulation of the cells. One possible explanation

could be that after seven days in primary culture bovine adrenocortical cells lose their ability to maintain the same levels of alpha-class GSTs, since there is well-documented evidence of such effects of aging in culture (for a review see **Hornsby *et al.*, 1979).**

The difference in effects observed for AII and ACTH are interesting since both these agonists are known to stimulate cortisol secretion in bovine adrenocortical cells on the days of culture used in these experiments. The main difference between the two agonists, as described in the introduction to this thesis, lies with the intracellular signalling system operating for each agonist. Whether this difference can partly or wholly explain the observed differences remains uncertain. Time-permitting, further experiments which might have been helpful here would involve stimulation of the cells with an anologue of cAMP, to stimulate the same effects of ACTH. Despite the fact that ACTH appears to decrease the mass of certain GSTs, it would be interesting to determine whether this steroidogenic agonist (or cAMP itself) is able to cause the phosphorylation of the GSTs known to be down-regulated. This follows from the findings of **Siegel *et al.*, (1990)** who confirmed earlier observations by **Pyerin *et al.*,(1987)** that several rat GST subunits (especially Ya and Yc) are substrates for protein kinase C. Such observations are obviously relevant only to agonists linked to phospholipase C such as AII (see "Introduction"), although it would be of interest to determine whether ACTH (or cAMP analogues) could cause similar phosphorylations via protein kinase A. Experiments involving Northern blot hybridisation could also be of use, involving analysis of mRNA levels corresponding to those enzymes thought to be down-regulated by ACTH. This method would enable the determination of whether the observed changes at the protein level were either pre- or post-translational.

The abundant expression of alpha-class GSTs in bovine adrenocortical cells is especially interesting in the light of the findings of **Rushmore *et al.*, (1991)**, in which an antioxidant responsive element was located in the region of DNA encoding for the rat GST Ya_2 subunit. Experiments here revealed hydrogen peroxide to produce a marked elevation in expression of the Ya subunit and, since evidence in this thesis suggests the alpha-class GSTs expressed in bovine adrenocortical cells are Ya-type, similar experiments involving hydrogen peroxide stimulation of bovine adrenocortical cells would also be of great interest.

The effects of ACTH observed in this preliminary study thus tend to support those of **Mankowitz *et al.* (1991a)** in which rat adrenocortical cells in primary culture showed down-regulation of GST following ACTH stimulation. However, the main GST affected in these studies belonged to the mu-class (Yb_2 subunit), which also showed a 15-fold elevation in expression during prolonged culture, in contrast to the bovine alpha-class GSTs. The reason for such differences is not clear, although the overall down-regulatory effect of ACTH appears very similar. The cell work described in this thesis focussed entirely on the alpha-class GSTs since these enzymes were found in such abundant amounts in this organ. In contrast, mu-class GSTs in the bovine adrenal cortex were found to be expressed at low levels in this organ, thuogh this does not preclude a physiological role. However, mu-class GSTs do not show significant peroxidase and isomerase activities, or activities with 4-hydroxyalkenals, and it is less clear why this GST class should show changes in expression levels during steroidogenesis in adrenocortical cells.

Concluding Remarks

Preliminary characterisation of GSTs from different bovine organs suggested these enzymes to be quite different to those in other species, and further studies, especially primary structure analysis, are clearly required to fully define bovine GSTs. Results from subsequent experiments on bovine adrenal cortex GSTs revealed remarkably high levels of alpha-class enzymes in this organ. Likewise, human adrenal cortex cytosol was found to contain high levels of an alpha-class GST which displayed many similar characteristics to the bovine enzymes, including glutathione peroxidase and Δ^5isomerase activities.

From such observations, it appears likely that alpha-class GSTs are important constituents of adrenocortical cells, probably involved in the detoxification of endogenous oxidative products formed during steroidogenesis or even in the steroid biosynthetic pathway itself (in the form of Δ^5-ketosteroid isomerase activity, for example). Further experiments on alpha-class GSTs in bovine organs also emphasised important zonal differences within the adrenal cortex. Immunohistochemical analysis of adrenal gland sections would be especially useful in confirming this observation, using antibodies specific for the different bovine alpha-class GSTs. Importantly, the abundant expression of alpha-class GSTs was found not to be unique to the adrenal cortex since both the liver and testes were shown to express similar levels of these enzymes. Since all these bovine organs (adrenal cortex, liver and testes) are capable of metabolising steroids, these findings emphasise the possible importance of alpha-class GSTs in steroid-metabolising organs. Observations made with human liver and adrenal cortex also support the findings in the bovine species.

Whatever the function of alpha-class GSTs in the adrenal cortex, their abundance argues for a fundamental role in the adrenocortical cell. Experiments involving the stimulation of bovine adrenocortical cells in primary culture with ACTH give further support to this theory whereby this steroidogenic agonist was found to down-regulate the alpha-class enzymes.

In summary, the results presented in this thesis describe the abundant expression of alpha-class GSTs in the adrenal cortex and suggest an important role for these enzymes in the normal metabolic functioning of this organ. This is another example of the growing awareness

of the importance of GSTs in endocrine organs and adds to the limited information currently existing on the expression of GSTs by various endocrine systems.

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Presentations/Publications Arising from Work in This Thesis.

(1) Abstract at 9th Joint Meeting of British Endocrine Societies (poster presentation)

"Isoenzymes of Glutathione-S-Transferase in Bovine Adrenal Cortex"

Meikle,I.,Bird,I.M.,Hayes,J.D.,and Walker,S.W. (1990)

J.Endocrinol., **124** (suppl), abstract 205.

(2) Abstract at 10th Joint Meeting of British Endocrine Societies (oral presentation):

"The GST Isoenzymes in Bovine Adrenal Cortex: Identification and Purification of an Abundant Subclass Exhibitting $^{5}\Delta$-3-ketosteroid Isomerase Activity."

Meikle, I., Hayes, J.D. and Walker, S.W. (1991)

J. Endocrinol., **129** (suppl), abstract 56.

(3) Meikle, I., Hayes, J.D. and Walker, S.W. (1991). Expression of an abundant α-class glutathione S-transferase in bovine and human adrenal cortex tissues.

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Expression of an abundant α-class glutathione S-transferase in bovine and human adrenal cortex tissues

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ABSTRACT

Bovine adrenal cortex tissue expresses high levels of glutathione S-transferase (GST) from each of the α, μ and π gene families. We describe the purification and characterization of an abundant α-class GST from this tissue that has not been identified previously because of its failure to bind to S-hexylglutathione–Sepharose 6B (S-hexG-Ag). This enzyme has been affinity purified on glutathione–Sepharose 6B (GSH-Ag) and was obtained in a highly purified form by employing S-hexG-Ag to remove the bulk of GST before chromatography on GSH-Ag. The purified GST eluted from GSH-Ag was found to exhibit marked peroxidase and Δ^5-ketosteroid isomerase activities (19·2 and 1·67 U/mg respectively). The bovine enzyme also showed high GST activity towards 4-hydroxynonenal (5·09 U/mg). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that the bovine GST contains two distinct polypeptides, one with an M_r of 25 900 and the other with an M_r of 26 500.

An abundant α-class GST was also purified from human adrenal cortex that possessed properties which were similar to the bovine α-class GST described above; however, unlike the bovine enzyme, the corresponding human α-class GST bound to S-hexG-Ag. As with the bovine enzyme, the purified human GST displayed marked peroxidase and isomerase activities (27 and 4·02 U/mg respectively). Further analysis on SDS-PAGE (M_r 25 800) and reverse-phase high-performance liquid chromatography established that this abundant α-class GST in human adrenal cortex is equivalent to the human liver GST B_1B_1 enzyme.

As both human and bovine adrenal cortex contain high levels of α-class GST with similar catalytic properties, we discuss the possible functions of these enzymes in this tissue.

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INTRODUCTION

The glutathione S-transferases (GSTs) are a complex group of detoxication enzymes that catalyse the formation of a thio-ether linkage between reduced glutathione (GSH) and a large spectrum of electrophiles including insecticides, herbicides, chemotherapeutic agents and carcinogens (Ketterer, 1986; Hayes & Wolf, 1988). This conjugation reaction is the first step in mercapturic acid synthesis (Chasseaud, 1979; Mannervik, 1985).

Multiple forms of GSTs exist, each with different catalytic and physicochemical properties. At least five distinct classes of GSTs have been identified that are encoded by separate families of genes, four of which are cytosolic and are called α, μ, π, and θ class GSTs (Mannervik, Ålin, Guthenberg *et al.* 1985; Hiratsuka, Sebata, Kawashima *et al.* 1990; Meyer, Coles, Pemble *et al.* 1991), the one remaining enzyme being a membrane-bound microsomal GST (Morgenstern & DePierre, 1983; McLellan, Wolfe & Hayes, 1989). The cytosolic GSTs are dimeric proteins and, whilst homodimers and heterodimers exist, each enzyme contains subunits which are members of the same gene family; in man, the α, μ, and π class GSTs comprise subunits of M_r 25 900, 26 600–26 700 and 24 800 respectively (Hayes, 1989).

Besides their ability to catalyse conjugation reactions, GSTs are now known to possess additional activities. Of interest here are the α-class GSTs, the diverse functions of which have been recognized for several years. For example, this class of GST is known to non-covalently bind non-substrate ligands (e.g. bile salts, bilirubin and penicillin) as well as binding covalently a number of carcinogens (e.g. 4-amino-azobenzene and 3-methylcholanthrene) (Litwack, Ketterer & Arias, 1971; Kirsch, Fleischner, Kamisaka & Arias, 1975; Ketterer, Tipping, Beale & Meuwissen, 1976; Hayes, Strange & Percy-Robb, 1979). Moreover, the α-class GSTs also exhibit other enzymic

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activities of potential physiological relevance such as selenium-independent glutathione peroxidase activity (Awasthi, Dao & Saneto, 1980) and Δ^5-ketosteroid isomerase activity (Benson, Talalay, Keen & Jakoby, 1977).

Certain GSTs are also known to detoxify epoxide derivatives of several environmental pollutants such as aflatoxin B_1 (Coles, Meyer, Ketterer *et al.* 1985; Hayes, Judah, McLellan *et al.* 1991). This mycotoxin, produced by the mould *Aspergillus flavus*, is converted to aflatoxin B_1 8,9-epoxide, which is known to be a potent hepatocarcinogen in certain species including the rat (Roebuck & Wogan, 1972). In this species α-class GSTs (e.g. Ya_1, Ya_2, Yc_1, Yc_2 and Yk subunits) have shown a marked increase in expression in livers possessing aflatoxin B_1-induced preneoplastic nodules, with the Ya_2 subunit displaying the greatest increase in concentration (Hayes, Kerr, Harrison *et al.* 1990; Hayes *et al.* 1991).

Importantly, the GSTs demonstrate marked organ-specific expression which is presumed to reflect differences in metabolic functions and physiological stresses encountered by different organs. In a previous study involving a number of different bovine organs, GST activity in the adrenal cortex was found to be exceeded only by that in the liver, further suggesting an important physiological role for the GSTs in this tissue (Hayes, Milner & Walker, 1989*b*). Synthesis of steroid hormones by the adrenal cortex requires the use of molecular oxygen with a resulting problem of toxicity caused by lipid peroxidation (Hornsby & Crivello, 1983*a*,*b*). The GSTs may be involved in combating this oxidative stress prevailing during steroidogenesis as several compounds generated in the process of lipid peroxidation have been found to act as substrates for GST, including 4-hydroxyalkenals (Ålin, Danielson & Mannervik, 1985), DNA hydroperoxides (Ketterer, Tan, Meyer & Coles, 1987) and cholesterol-α-oxide (Meyer & Ketterer, 1982). Furthermore, previous studies have shown that α-class GSTs, in addition to the selenium-dependent form of glutathione peroxidase, are able to inhibit lipid peroxidation *in vitro* (Tan, Meyer, Belin & Ketterer, 1984). The potential importance of GST in helping combat oxidative stress is also emphasized by the recent observation that the 5′ flanking region of one of the rat α-class GST genes (i.e. that encoding the Ya_2 subunit) contains an antioxidant-responsive element (ARE) which results in transcriptional activation of GST during oxidative stress (Rushmore & Pickett, 1990; Rushmore, Morton & Pickett, 1991).

In this present investigation we have studied the properties of the GSTs expressed in both bovine and human adrenal cortex tissues. After purification by affinity chromatography we demonstrate that the most abundant GST expressed in these tissues belongs to the α-class. The physiological significance of this finding is discussed and the possible functions of α-class GSTs in the adrenal cortex are considered.

MATERIALS AND METHODS

Chemicals

S-hexylglutathione was synthesized by the method of Vince, Daluge & Wadd (1971). The S-hexylglutathione–Sepharose 6B (S-hexG-Ag) affinity gel was constructed by using the method of Mannervik & Guthenberg (1981). Glutathione–Sepharose 6B (GSH-Ag) was purchased from Sigma Chemical Co., Poole, Dorset, U.K. The Δ^5-androstene-3,17-dione was a gift from Dr P. K. Stockman (Royal Infirmary, Edinburgh). 4-Hydroxynonenal was a gift from Professor H. Esterbauer (Department of Biochemistry, University of Graz, Graz, Austria). All other chemicals were of analytical grade and readily available commercially. The high-performance liquid chromatography (HPLC) solvents were from Rathburn Chemicals, Walkerburn, Peeblesshire, U.K.

Buffers

All buffers were prepared at room temperature, although the pH values quoted are those obtained at the temperature at which they were used (e.g. 4 °C).

Tissue

Bovine adrenal glands were obtained from a local slaughterhouse within 30 min of death and transported to the department on ice. On arrival the fat was trimmed and adrenal cortex and medulla manually dissected from one another before storing the cortex tissue at −85 °C.

The human adrenocortical tissue was a right adrenal gland obtained at operation from a patient with bilateral adrenal hyperplasia due to Cushing's disease. The tissue was obtained immediately after surgery and stored at −85 °C until use.

Enzyme purification

Preparation of cytosol

Homogenates (1:4 (w/v)) of tissues from both species were prepared in ice-cold Tris–HCl buffer (50 mmol/l; pH 7·8) that contained 200 mmol NaCl/l (buffer A). Standard centrifugation techniques were used to prepare 100 000 *g* supernatant fractions (cytosol), which were subsequently dialysed for 16 h at 4 °C against 4 litres buffer A containing 1 mmol 2-mercaptoethanol/l

(buffer B). After dialysis, the cytosol was re-centrifuged at 15 000 *g* for 30 min at 4 °C to remove precipitated material, and the resulting supernatant subjected to affinity chromatography.

Isolation of GST

Enzymes were prepared from each of the cytosols by affinity chromatography on both S-hexG-Ag and GSH-Ag which had been equilibrated at 4 °C with buffer B.

Preliminary experiments using cytosol prepared from bovine adrenal cortex had shown that the major α-class GSTs failed to bind to S-hexG-Ag. Hence, S-hexG-Ag was used as a preliminary purification step only. Dialysed cytosol (500 ml; 4 g protein) was applied initially to the S-hexG-Ag column (1·6 × 30 cm) and the flow-through from this column then re-applied to the GSH-Ag column. Non-specifically adsorbed protein was removed by extensive washing with buffer B (10 column volumes), because specifically bound material was eluted using a solution of 40 mmol GSH/l in 200 mmol Tris–HCl buffer/l (pH 9·0).

In the case of cytosol prepared from human adrenal cortex, it was not known whether the α-class GSTs adsorb to S-hexG-Ag or behave like the major α-class GST pool from the bovine adrenal cortex and fail to bind to this matrix. Hence, GSTs were eluted from both S-hexG-Ag and GSH-Ag; these two columns were used sequentially, as described above for bovine adrenal cortex preparations. Dialysed human adrenal cortex cytosol (25 ml; 0·35 g protein) was therefore applied to the S-hexG-Ag matrix initially (1 × 4 cm) and, after extensive washing with buffer B, the specifically bound protein was eluted with a solution of 5 mmol S-hexylglutathione/l in buffer A. As with the bovine cytosol, flow-through from the S-hexG-Ag column was then applied to a column (1 × 4 cm) containing GSH-Ag, and specifically bound protein was eluted as described above.

Enzyme and protein determination

All enzyme assays were carried out at 37 °C. Assays with 1-chloro-2,4-dinitrobenzene as the substrate were carried out using the method described by Habig & Jakoby (1981). Selenium-independent glutathione peroxidase activity towards cumene hydroperoxide was determined using the coupled assay system of Reddy, Tu, Burgess *et al.* (1981). Both of these enzyme assays were performed on a Cobas Fara centrifugal analyser (Roche Diagnolstics, Welwyn Garden City, Herts, U.K.) as were measurements of protein concentrations, using the dye-binding method of Bradford (1976).

Enzymic activities using Δ^5-androstene-3,17-dione and 4-hydroxynonenal as substrates were determined manually as described by Benson & Talalay (1976) and Ålin *et al.* (1985) respectively.

Electrophoretic methods

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% (w/v) polyacrylamide resolving gels that contained 0·32% (w/v) methylenebisacrylamide (for details see Hayes & Mantle, 1986). Rat liver GST subunits were employed as M_r standards: Ya (M_r 25 500), Yb (M_r 26 300) and Yc (M_r 27 500 (Hayes, 1988).

Reverse-phase HPLC

Linear 40–58% gradients of acetonitrile in aqueous 0·1% (v/v) trifluoroacetic acid were used. Details of the HPLC system employed and other experimental details have been described previously (Hayes, Kerr & Cronshaw, 1989*a*).

RESULTS

Purification and characterization of bovine adrenal cortex GST

Both the affinity matrices S-hexG-Ag and GSH-Ag were used to purify GST from bovine adrenal cortex tissue cytosol. Affinity chromatography of GST from this tissue on S-hexG-Ag has been characterized previously (Hayes *et al.* 1989*b*). Analysis of the eluate from this column showed that significant GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) was recovered in the 'flow-through' fractions (Table 1), suggesting that some GSTs were not binding to this affinity matrix. The use of GSH-Ag as an additional matrix allowed the GSTs which were not adsorbed by the S-hexG-Ag column to be purified. These fractions were analysed by SDS-PAGE (Fig. 1). This demonstrated that bovine adrenal cortex cytosol (lane 2) contained an abundant protein(s), which had a similar electrophoretic mobility to rat GST Ya, Yb and Yc subunits, and, furthermore, that this protein was also observed in the flow-through fractions from the S-hexG-Ag column (lane 3). However, the material which failed to bind to GSH-Ag (lane 4), after application of S-hexG-Ag, did not appear to show the corresponding protein bands, and is consistent with the GSH-Ag column having successfully removed these subunits from the material recovered in the S-hexG-Ag flow-through fraction.

The enzyme activities using CDNB as a substrate (Table 1) revealed that about 35% of the total CDNB activity remained in the flow-through from the S-hexG-Ag column, but that this activity was greatly reduced in the flow-through fraction after its application to GSH-Ag. An identical situation was found

TABLE 1. Glutathione S-transferase (GST) activities of bovine adrenal cortex cytosols at different stages of affinity chromatography, as well as activities of the purified GST pool, using different model substrates

	GST substrate (μmol/min per mg protein)			
	CDNB	CuOOH	ADD	OH-NON
Pre-column cytosol	0·24	0·23	0·015	0·065
S-hexG-Ag flow-through	0·09	0·26	0·014	0·061
GSH-Ag flow-through	0·01	0·13	0·001	NS
GST pool purified on GSH-Ag	9·59	19·2	1·67	5·09

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5-androstene-3,17-dione; OH-NON, 4-hydroxynon-2-enal; S-hexG-Ag, S-hexylglutathione–Sepharose 6B; GSH-Ag, glutathione–Sepharose 6B; NS, not significant.
The coefficient of variation obtained using the centrifugal analyser (i.e. CDNB and CuOOH assays) was less than 5%; for the ADD assay this value was 7·5%, and for the OH-NON assay 6·5%.

when cumene hydroperoxide, Δ^5-androstene-3,17-dione and 4-hydroxynonenal were used as substrates. These substrates show high activity with certain α-class GSTs, and this result suggests that the GST purified on the GSH-Ag column was a member of this family. The peroxidase activity remaining in the flow-through from the second column could be attributed to the selenium-dependent enzyme (GPx), which shows activity with cumene hydroperoxide and does not bind to either affinity matrix used in this study.

The GST isoenzymes binding to the GSH-Ag column were eluted using 40 mmol GSH/l, and the GST activity towards CDNB and protein concentration in each fraction determined. Those fractions contributing to the corresponding peak of activity were pooled (results not shown). SDS-PAGE analysis (Fig. 1) showed this pool to consist of two distinct bands of unequal staining intensity (lanes 5 and 6): a more densely staining, faster-migrating band of M_r 25 900, and a less densely staining, slower-migrating band of M_r 26 500. The M_r 25 900 band represented a GST polypeptide which is particularly abundant in adrenal cortex cytosol and is estimated to comprise 1·3% of the cytosolic protein in this organ.

The affinity-purified pool was found to be moderately active with CDNB as a substrate, although it showed relatively high activity with cumene hydroperoxide, Δ^5-androstene-3,17-dione and 4-hydroxynonenal as substrates (Table 1).

Comparison of GST expression in bovine adrenal cortex with human liver and human adrenal cortex

To help evaluate the significance of high levels of α-class GST in the bovine adrenal cortex, GST expression in human adrenal cortex was also examined. Human liver GSTs were included in this study to provide a source of enzyme which has been characterized previously and could, therefore, serve as a protein standard.

Cytosols from each of the above three tissues showed evidence of abundant levels of proteins in the GST region during SDS-PAGE analysis (Fig. 2). Bovine adrenal cortex cytosol (lane 4) showed the two bands described above, whilst both human liver and human adrenal cortex (lanes 2 and 3 respectively) also showed abundant levels of one band in this region. This latter human polypeptide band had a similar electrophoretic mobility to the fast-migrating bovine α-class GST subunit (M_r 25 900) described above. Furthermore, comparison with the GST B_1B_1 standard in lane 5 demonstrates that the abundant electophoretic band in both human liver and adrenal cortex had identical mobility and may therefore be identical to this α-class GST.

Purification and characterization of human adrenal cortex GST

The GSTs in cytosol prepared from human adrenal cortex tissue were purified on S-hex-Ag (Fig. 3). The abundant protein found in the GST region of cytosol from this tissue on SDS-PAGE (lane 2) was removed following affinity chromatography on S-hexG-Ag, with no obvious GST remaining in the flow-through from this column (lane 3). As expected from this result, application of flow-through from the S-hexG-Ag column to GSH-Ag did not result in the purification of further GSTs (lane 4).

These observations are also supported by measurement of GST activity at different stages of purification using different substrates (Table 2). Clearly, almost all activity (using CDNB as a substrate) was removed from the cytosol during the first stage of affinity chromatography on S-hexG-Ag, with very little remaining in the flow-through from either affinity column. Similarly, using both cumene hydroperoxide and Δ^5-androstene-3,17-dione as substrates, activity was almost completely absent in the flow-through from the S-hexG-Ag column. Hence, unlike bovine adrenal cortex, α-class GST activity appeared to be retained on S-hexG-Ag using cytosol from human adrenal cortex.

TABLE 2. Glutathione S-transferase (GST) activities of human adrenal cortex cytosols at different stages of affinity chromatography, as well as activities of the purified GST pool, using different model substrates

	GST substrate (μmol/min per mg protein)		
	CDNB	CuOOH	ADD
Pre-column cytosol	0·64	0·34	0·075
S-hexG-Ag flow-through	0·06	0·08	NS
GSH-Ag flow-through	0·03	0·07	NS
GST pool purified on S-HexG-Ag	73	27	3·97
Human Liver B_1B_1	82*	31*	4·02

*Data for these substrates are taken from Stockman, McLellan & Hayes (1987).
Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; ADD, Δ^5-androstene-3,17-dione; S-hexG-Ag, S-hexyl-glutathione–Sepharose 6B; GSH-Ag, glutathione–Sepharose 6B; NS, not significant.
The coefficient of variations for each assay were as for Table 1.

2). This is consistent with the findings from SDS-PAGE that the abundant GST subunit in human adrenal cortex belongs to the α-class. Further comparison of specific enzyme activities for the human adrenal cortex GST with those of human liver GST B_1B_1 revealed a marked similarity between the two enzymes (see Table 2 for comparison).

Reverse-phase HPLC of human adrenal cortex GST

Application of the human adrenal cortex GST pool to a reverse-phase HPLC system followed by gradient elution led to the appearance of one main peak (Fig. 4*b*). Previous application of human liver GST B_1B_2 established that the B_1 subunit co-eluted with this single peak of adrenal GST (Fig. 4*a*). Simultaneous application of the human adrenal cortex GST pool with human liver GST B_1B_2 produced a marked increase in the B_1 peak (Fig. 4*c*), again consistent with this GST subunit, abundantly expressed in human adrenal cortex cytosol, being identical to the B_1 subunit expressed in human liver cytosol.

DISCUSSION

Bovine GSTs have not been well characterized, and existing information in the literature is limited (Asaoka, 1984; Schaffer, Galley & Ladenstein, 1988; Young & Briedis, 1989). Even less is known about the expression of GST in the adrenal cortex of cattle or other species (including man), despite the fact that the adrenal cortex is known to have high levels of glutathione (Hornsby & Crivello, 1983*b*). Work carried out in this laboratory has shown the bovine adrenal cortex expresses subunits from the three α, μ, and π classes of GST (Hayes *et al.* 1989*b*). During the course of further work on GSTs in bovine adrenal cortex, we have purified an abundant α-class GST using GSH-Ag as an alternative affinity matrix. This GST pool failed to bind to S-hexG-Ag, a phenomenon initially observed for the rat α-class Yk subunit (Hayes, 1986), although it has since been found for other GST subunits (Hayes, 1988).

Unlike the major bovine α-class GST, the human α-class enzyme was purified on S-hexG-Ag. The reason for such a marked difference in chromatographic behaviour is unclear but it suggests that structural differences exist between the α-class enzymes from the two species, even though their catalytic properties are closely similar (Tables 1 and 2). An obvious difference between the α-class enzymes from both species is the presence of an additional, slower-migrating polypeptide (M_r 26 500) observed during SDS-PAGE analysis of the bovine enzyme. The corresponding electrophoretic band is not apparent during SDS-PAGE analysis of bovine adrenal cortex GST purified on S-hexG-Ag (Hayes *et al.* 1989*b*), suggesting it to be similar to the Yk subunit (Hayes, 1986). This does not, however, explain the lack of affinity of the faster-migrating polypeptide (M_r 25 900) towards S-hexG-Ag, which could be attributed to species differences between the bovine and human α-class enzymes.

The GST purified on GSH-Ag demonstrated all the characteristics of an α-class GST, including migration position on SDS-PAGE and activity towards different model GST substrates. Of particular interest was the fact that the purified transferase enzyme exhibited both marked selenium-independent glutathione peroxidase and Δ^5-ketosteroid isomerase activities. Since the purified enzyme pool was shown to consist of at least two bands by SDS-PAGE (M_r 25 900 and 26 500), it is possible that the different subunits display different substrate activities; this question is currently under investigation.

The abundant α-class GST purified from human adrenal cortex cytosol on S-hexG-Ag was further shown to be equivalent to the human liver GST B_1 α-class subunit on the basis of specific activities with different model GST substrates and co-migration by reverse-phase HPLC. Like the major bovine adrenal cortex GST, the purified human enzyme also showed high selenium-independent glutathione peroxidase and Δ^5-ketosteroid isomerase activities. The possible significance of these substrate activities is discussed below.

The abundance of α-class GSTs in cytosol from bovine and human adrenal cortex tissues is striking. Indeed, cytosols prepared from cultured bovine adrenocortical cells show that the main α-class GST bands stain more intensely with Coomassie blue than any other cytosolic protein. This intense staining is

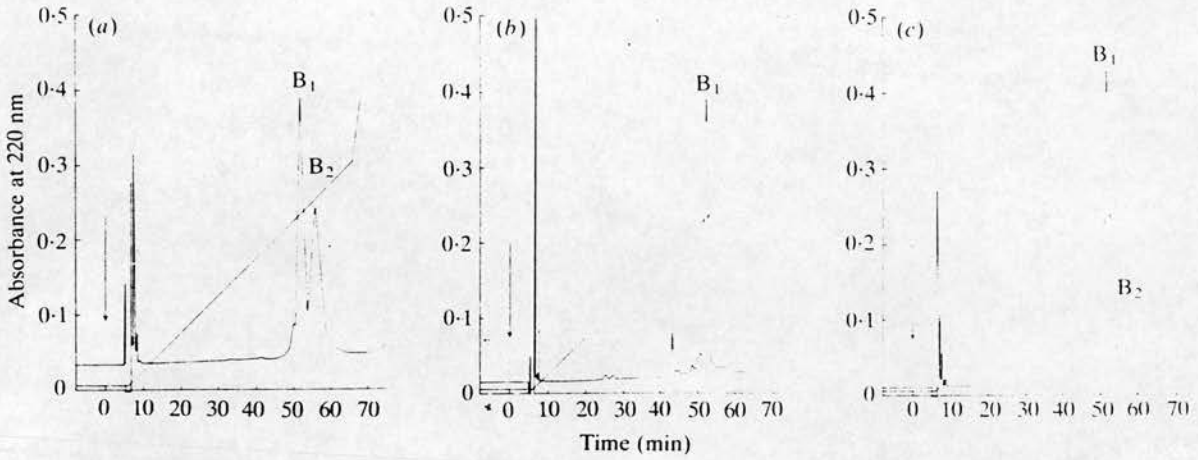

FIGURE 4. Identification of human adrenal cortex glutathione S-transferase (GST) by reverse-phase HPLC. The abundant GST expressed in human adrenal cortex tissue cytosol was identified as being equivalent to human liver GST B_1B_1 by sequentially running (*a*) human liver GST B_1B_2, (*b*) human adrenal cortex GST purified on S-hexylglutathione–Sepharose 6B and (*c*) human adrenal cortex GST plus human liver B_1B_2. The column employed was a Waters μBondapak C_{18} column (10 μm particle size; column size 0·39 × 30 cm) which was developed at 1 ml/min using a linear 40–58% acetonitrile gradient in aqueous 0·1% trifluoroacetic acid formed over 60 min. This was followed by a 58–70% acetonitrile gradient in aqueous 0·1% trifluoroacetic acid formed over 5 min. The eluate was monitored at 220 nm. The arrows indicate the time of loading the sample on to the column and commencement of the 5-min loading time before initiation of the gradient; pump A delivered 40% acetonitrile and pump B 70%.

reflected in the abundant protein yield from whole tissue and suggests a fundamental role for this α-class GST in the function of bovine and human adrenal cortex. The activities of the enzymes in both species towards cumene hydroperoxide (i.e. selenium-independent glutathione peroxidase activity) and Δ^5-androstene-3,17,-dione (i.e. Δ^5-3-ketosteroid isomerase activity) is particularly noteworthy.

Lipid peroxidation and other aspects of oxygen toxicity are a potential problem for adrenal cortex tissue, and the presence of a high peroxidase activity to overcome such oxidative stress would seem appropriate. A possible function *in vivo* for the α-class GSTs described above may be, therefore, to prevent adrenocortical cell damage caused by lipid hydroperoxides and other free oxygen radicals formed as by-products during the synthesis of steroids. Further results which support this theory come from enzyme activities with a group of compounds called 4-hydroxy-2,3-*trans*-alk-2-enals, first described as substrates for GSTs by Ålin *et al.* (1985). The 4-hydroxyalkenals are known to occur in the cell as a result of oxidative metabolism of endogenous, as well as foreign, compounds, and studies have shown relatively large amounts to be produced during stimulated lipid peroxidation (Esterbauer, Cheeseman, Dianzani *et al.* 1982; Poli, Dianzani, Cheeseman *et al.* 1985). The bovine α-class GST pool purified in this study was found to exhibit marked activity with 4-hydroxynonenal as a substrate. This finding, in addition to the peroxidase activity, lends further support to the idea that the bovine α-class GST described above is involved in the detoxification of endogenous oxidized products formed during steroidogenesis. In terms of species differences, the specific activity using 4-hydroxynonenal with the bovine enzyme is significantly greater than that quoted for the human B_1B_1 enzyme (Danielson, Esterbauer & Mannervik, 1987). It is possible to speculate that the extra band present in the bovine α-class pool may be responsible for the 4-hydroxyalkenal activity, since the single GST band from the human tissue displayed both marked peroxidase and isomerase activities (greater than bovine) although an apparently low hydroxyalkenal activity. It is relevant to note that the bovine adrenal cortex is known to be remarkably well served by a number of biological antioxidants (for review see Hornsby & Crivello, 1983*b*), and the role of α-class GSTs may only be as part of an overall antioxidant system.

The marked Δ^5-ketosteroid isomerase activity exhibited by the purified α-class enzyme pool from both species may also be significant in terms of adrenocortical function. This isomerization reaction occurs at a number of stages during the synthesis of steroids, where it is assigned to the microsomal 3-β-steroid dehydrogenase/Δ^5-ketosteroid isomerase enzyme (Naville, Rainey, Milewich & Mason, 1991). The significance of the relatively high activity shown by the α-class enzymes from both species for the ring isomerization might suggest that they have an integral

role in the steroid biosynthetic pathway. At present, this remains speculative, but further experiments are in progress to test the substrate specificity and kinetic characteristics of this reaction in more detail.

In summary, we have described the abundant expression of α-class GSTs in both bovine and human adrenal cortex cytosols. Specific enzymic activities with known α-class substrates have indicated potential roles for these enzymes *in vivo*, although further experiments are clearly required to verify such suggestions. Preliminary work in this laboratory has revealed that, of all other bovine tissues studied so far, the abundance of an α-class GST showing low affinity for S-hexG-Ag (as described for the adrenal cortex) is observed only in the liver and testes. This feature of GST expression in cattle may be characteristic of steroid-metabolizing tissue, and future experiments aim to establish a role(s) for these enzymes in such organs.

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